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THE TRICARBOXYLIC ACID AND GLYOXYLATE CYCLES IN XANTHOMONAS PHASEOLI (XP8)[1]

N. B. MADSEN AND R. M. HOCHSTER

Abstract

Cell-free extracts of *Xanthomonas phaseoli* contain the individual enzymes of the tricarboxylic acid cycle, and it is suggested that this is the main pathway for the terminal oxidation of carbohydrate in this organism. *X. phaseoli* can grow on a medium containing acetate as the sole source of carbon. Cell-free extracts of such acetate-grown organisms contain the enzymes of the glyoxylate cycle, and it is concluded that the operation of this cycle permits the initial stages of synthesis of complex cell material from acetate at a rate sufficiently high to account for the observed rate of growth on the acetate medium. The two enzymes required to modify a tricarboxylic acid cycle into a glyoxylate cycle are present in very small amounts (malate synthetase) or absent entirely (isocitritase) in extracts of glucose-grown *X. phaseoli*.

Introduction

The tricarboxylic acid cycle is widely accepted as being the common "terminal" pathway of oxidation of all foodstuffs. The importance of this cycle in the energy metabolism of organisms may be seen from the fact that two thirds of the energy available from the oxidation of glucose is derived from its operation. This statement applies where either the glycolytic scheme or the "hexose cycle" proposed by Hochster and Katznelson (4) is used to break glucose down to the pyruvate stage, but does not apply where the glucose is oxidized partially or completely through the mediation of the pentose cycle. The tricarboxylic acid cycle does more than merely provide energy; some of its intermediates provide carbon skeletons for the synthesis of amino acids. When organisms grow on complex substrates, carbon dioxide fixing reactions, such as form malate from pyruvate, are thought to replenish the supply of intermediates drained from the cycle. Such reactions cannot, however, explain how organisms can grow on simple substrates such as acetate or ethanol, because, as may be seen in Fig. 1 below, the operation of the tricarboxylic acid cycle leads to the complete oxidation of acetate and does not allow for the net formation of any of the intermediates.

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To explain the phenomenon of growth on acetate as sole carbon source, the "glyoxylate cycle" has been invoked by Kornberg and Madsen (12, 13, 14), and has been discussed in the review by Kornberg and Kreds (11). As may be seen in Fig. 1, the operation of this cycle leads to the net production of one molecule of a four-carbon dicarboxylic acid from two molecules of acetate. This cycle has been found in a number of microorganisms (10, 14), and in castor beans (9), but could not be demonstrated in certain animal tissues (15).

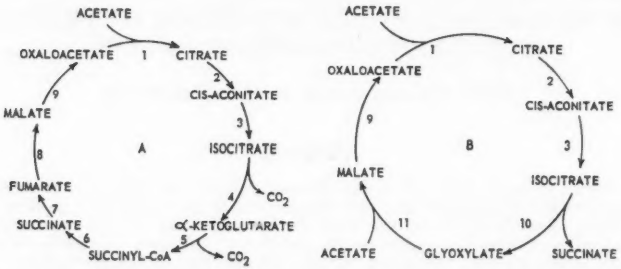

FIG. 1. Schematic representation of the tricarboxylic acid cycle (A) and the glyoxylate cycle (B).

Previous work in this laboratory has been concerned with the mechanism of the oxidation of glucose to the pyruvate level in extracts of the plant pathogen *X. phaseoli* (Hochster and Katznelson (4)). These authors also showed that cell-free extracts of glucose-grown *X. phaseoli* could oxidize intermediates of the tricarboxylic acid cycle, and more recent work has shown that extracts of acetate-grown cells can also oxidize these intermediates, though at a slower rate. Work reported in this paper was initiated to determine whether the terminal oxidation pathway in this organism follows the conventional tricarboxylic acid cycle, and to establish the possible role played by the glyoxylate cycle.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ATP,* AMP, TPN, DPN, and CoA (Pabst Brewing Co.); α-ketoglutaric acid, lipoic acid, phenazine methosulphate (Sigma Chemical Co.); glyoxylic acid, isocitric acid, *cis*-aconitic acid (California Foundation for Biochemical Research); ThPP, oxaloacetic acid, fumaric acid, *l*-malic acid, pyruvic acid (Nutritional Biochemicals Corporation); GSH (Schwarz Laboratories); succinic acid, citric acid (Fisher Scientific Co.); and sodium acetate-2-C^{14} (Merck & Co.).

*The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced form of triphosphopyridine nucleotide; ThPP, thiamine pyrophosphate; GSH, reduced glutathione, CoA, coenzyme A.

Methods

X. phaseoli (XP8) was grown in 2800-ml Fernbach flasks and incubated on a rotary shaker at 25 to 30°. "Glucose-grown" cells were produced in a medium containing 1% yeast extract and 2% glucose; 50 ml of this medium was inoculated from a yeast–glucose–$CaCO_3$–agar slant culture and incubated for 18 to 24 hours; the 50-ml inoculum thus obtained was then added to 700 ml of medium contained in the Fernbach flasks and the latter incubated for 18 to 24 hours.

"Acetate-grown" cells were produced in a medium containing 26 millimoles (mM) of ammonium acetate, 36 mM of phosphate buffer at pH 7.2, 1 mM of $MgSO_4$, and 9.6 ml of trace elements solution* per 1000 ml. The culture was maintained by successive transfers through 50-ml aliquots of this medium, shaken in 300-ml Erlenmeyer flasks. For large-scale growth, 700 ml of the medium was inoculated with a 50-ml culture and incubated for 3 days.

Cells were harvested by centrifugation at 4000 r.p.m., washed twice with 0.9% NaCl, and suspended in two volumes of 0.1 *M* potassium phosphate buffer (pH 7.5) containing 0.01 *M* GSH. They were then treated with sonic vibration in a Raytheon 200-watt, 10-kc oscillator for 15 minutes at a power output of 1.0 amp. The suspension was centrifuged at 11,000 r.p.m. (15,000×*g*) at 5° for 10 minutes and the supernatant solution poured off and used.

For experiments on the incorporation of C^{14} from acetate-2-C^{14}, the incubation mixture used contained 100 micromoles (μM) of potassium phosphate, pH 7.5, 10 μM of GSH, 10 μM of $MgCl_2$, 0.13 μM of CoA, 10 μM of ATP, 2 μM of acetate-2-C^{14} (containing 10 μc and giving 336,000 counts/minute under the conditions of assay), 3 μM of unlabelled potassium acetate, 10 μM of other unlabelled substrates, as indicated, and water to 0.9 ml. The mixture was incubated for 2 to 3 minutes at 30°, 0.1 ml of extract containing 6 mg of protein was added, and incubation continued for 30 minutes. The unlabelled substrates oxaloacetate, glyoxylate, pyruvate, and α-ketoglutarate were each added in five equal aliquots at 6-minute intervals to their respective tubes. After 30 minutes of incubation, 3 ml of hot ethanol were added to the tubes, the precipitate was centrifuged off and washed with 1 ml of 70% ethanol. The combined supernatant solutions were evaporated to dryness at 60° under a stream of N_2 gas. The residues were dissolved in 0.4 ml of H_2O and aliquots (0.1 ml) were applied to the "origin" positions (10 cm in from each side of one corner) of Whatman No. 4 chromatographic paper sheets (22 in. × 20 in.). They were then subjected to chromatographic analyses, radioautography, and radioassay according to the methods described in detail by Kornberg (8). In brief, the method consisted of two-dimensional paper chromatography, location of the radioactive compounds by exposure to X-ray film for 1 week, and counting of the radioactive area directly on the paper with a mica end-window β-counter tube.

*The trace elements solution contained, per liter, the following substances: 10 ml of 1 *N* HCl, 5.1 mg of H_3BO_3, 52 mg of $CaCO_3$, 10 mg of $CuSO_4.5H_2O$, 100 mg of $FeSO_4$, 1 mg of KI, 20 mg of $MnSO_4.H_2O$, 17 mg of $Na_2MoO_2.2H_2O$, and 51 mg of $ZnSO_4.7H_2O$.

Protein concentration was estimated by the spectrophotometric method of Warburg and Christian (21). Alpha-keto acids were measured as the 2,4-dinitrophenylhydrazones by the procedure of Friedemann and Haugen (3). The 2,4-dinitrophenylhydrazones were chromatographically identified by the method of El Hawary and Thompson (2). The activities of several enzymes were estimated by measuring the reduction of phosphopyridine nucleotides at 340 mμ in the Beckman spectrophotometer (Model DU) at 22 to 24° or at 30°, as detailed in the text. For the determination of α-ketoglutaric dehydrogenase, Beckman corex cells contained: 100 μM of potassium phosphate, pH 7.5, 2 μM of $MgCl_2$, 10 μM of GSH, 0.3 μM of lipoic acid, 0.3 μM of ThPP, 0.6 μM of DPN, 0.4 μM of CoA, 25 μM of α-ketoglutaric acid, 0.1 ml of extract, and distilled water to 3.0 ml. The reaction was started by the addition of either α-ketoglutaric acid or CoA, and the reduction of DPN was followed at 340 mμ.

P enzyme, which catalyzes the reaction,

$$\text{succinyl-CoA} + \text{ADP} \leftrightharpoons \text{succinate} + \text{ATP} + \text{CoA},$$

was measured in the direction of succinyl-CoA formation in the presence of hydroxylamine by the method of Kaufman (6). Succinic dehydrogenase was measured by the manometric procedure of Singer and Kearney (19) in which the dye, phenazine methosulphate, is employed. Fumarase activity was measured by the method of Massey (16), by following the decrease in optical density at 300 mμ as an indication of the hydration of the double bond in fumarate.

Malic dehydrogenase activity was determined in Beckman corex cells containing: 100 μM of potassium phosphate, pH 7.5, 180 μM of sodium cyanide (to bind the oxaloacetate formed and so to shift the equilibrium in the direction of oxaloacetate formation), 600 μM of potassium malate, 0.6 μM of DPN, 0.1 ml of cell-free extract, and water to 3.0 ml. The reduction of DPN was followed at 340 mμ.

Results

Using the method of Jones and Lipmann (5), the cell-free extracts were found to activate acetate in the presence of added coenzyme A, glutathione, and ATP, to form acetyl-coenzyme A, which was detected in the assay by the formation of hydroxamic acid. Extracts from acetate-grown cells activated 1.2 μM of acetate per hr per mg of protein, whereas the rate in extracts from glucose-grown cells was only one fifth of this, 0.25 μM per hr per mg of protein. The presence of the acetate-activating enzyme (aceto-CoA-kinase) made it possible to study the incorporation of radioactive acetate into various compounds of the tricarboxylic acid cycle.

When acetate-2-C^{14} was added to cell-free extracts in the presence of added ATP, coenzyme A, and glutathione, C^{14} was incorporated chiefly into acetyl-coenzyme A, with traces in malate, fumarate, and citrate or isocitrate (Table I).

The addition of oxaloacetate (line *d*) to such a reaction mixture resulted in the heavy incorporation of acetate-2-C^{14} into citrate, indicating the presence of condensing enzyme (Ochoa, Stern, and Schneider (17)) (reaction 1, Fig. 1). The lower incorporation by extracts from glucose-grown cells is not necessarily significant because aceto-CoA-kinase is limiting here.

TABLE I

Incorporation of acetate-2-C^{14} in the presence of various tricarboxylic acid cycle intermediates

Tube	Compound added	Extract from acetate-grown cells, radioactivity (counts/min) in:			Extract from glucose-grown cells, radioactivity (counts/min) in:		
		Malate	Fumarate	Citrate or isocitrate	Malate	Fumarate	Citrate or isocitrate
a	None	90	40	90	90	—	1,570
b	Glyoxylate	42,840	7,300	1,670	1,470	230	2,670
c	Isocitrate	920	60	320	60	50	820
d	Oxaloacetate	20	50	42,500	—	50	19,500
e	Malate	80	80	1,470	230	120	9,370
f	Citrate	70	—	50	70	90	780
g	α-Ketoglutarate	—	50	450	40	80	1,840
h	Pyruvate	70	30	2,450	—	60	6,300

The presence of malate synthetase (reaction 11, Fig. 1) (Wong and Ajl (22)) in the extracts of acetate-grown cells is indicated by the large incorporation of radioactive acetate into malate when glyoxylate is added to the incubation mixture (line *b*, Table I). The glucose-grown organisms also contain malate synthetase but the results show that it is present in much smaller amounts.

The incorporation of acetate into malate in the presence of isocitrate (line *c*, Table I) shows that the acetate-grown organisms contain the enzyme isocitritase whereas the glucose-grown organisms do not. The action of isocitritase (reaction 10, Fig. 1) (Campbell, Smith, and Eagles (1), Smith and Gunsalus (20)) splits isocitrate into succinate and glyoxylate, and the latter then combines with acetyl-coenzyme A to form malate.

The increased incorporation of acetate-C^{14} into citrate in the presence of malate, α-ketoglutarate, or pyruvate suggests that the enzymes necessary to convert these compounds to oxaloacetate are also present.

Table II lists the enzymes of the tricarboxylic acid and glyoxylate cycles, together with their specific activities in extracts from acetate- and glucose-grown *X. phaseoli* cells. The extracts contain both a TPN-specific and a DPN-specific isocitric dehydrogenase (reaction 4, Fig. 1) as measured by the reduction of the respective nucleotides in the presence of isocitrate. The DPN-specific dehydrogenase differed from the yeast enzyme in that it was not activated by adenosine monophosphate or inhibited by cyanide (7). It was possible to measure aconitase (reactions 2 and 3, Fig. 1) by the reduction of TPN when isocitrate was produced from either citrate or *cis*-aconitate. The rate with the latter substrate is shown in Table II. Proof that the reduction of TPN was a function of aconitase acting in conjunction with isocitric dehydrogenase was provided by chromatographic identification of

α-ketoglutaric acid in the reaction mixture, as well as by the colorimetric measurements of this product which showed that is was formed in amounts approximately equal to the TPN reduced (Table III).

The presence of an active isocitric dehydrogenase also made it possible to measure the rate of formation of isocitrate from succinate and glyoxylate by the action of the enxyme isocitritase, by following the reduction of TPN in the spectrophotometer as described by Kornberg and Madsen (14). Isocitritase was found to be present in extracts from acetate-grown cells, but it could not be detected in extracts from glucose-grown cells. This enzyme was also measured (by a modification of the method of Saz and Hillary (18)) in the reverse direction by determining the rate of formation of glyoxylic acid from isocitrate. Again the acetate-grown cells had considerable activity but none could be detected in the glucose-grown cells. In both cases the reaction

TABLE II

Reaction rates of enzymes of the tricarboxylic acid and glyoxylate. Cycles in cell-free extracts of *Xanthomonas phaseoli*

Reaction numbered as in Fig. 1	Name of enzyme	Reaction rate, μM of substrate per hr per mg protein	
		Acetate-grown cells	Glucose-grown cells
	Aceto-CoA-kinase	1.2	0.2
1	Condensing enzyme	0.8	Present†
2 and 3	Aconitase	0.7*	10.0*
4	Isocitric dehydrogenase (TPN)	2.8*	17.0*
4	Isocitric dehydrogenase (DPN)	0.9*	5.0*
10	Isocitritase (in direction of splitting)	0.6	0.0
10	Isocitritase (in direction of synthesis)	1.1*	0.0*
11	Malate synthetase	1.1	0.03
5	α-Ketoglutaric dehydrogenase	0.6	0.7
6	P enzyme	1.0	1.2
7	Succinic dehydrogenase	1.0	1.8
8	Fumarase	26.0	28.0
9	Malic dehydrogenase	2.1	2.3

NOTE: Reactions were run as described in the text at 30°, except those with *.
*Reactions were run at 22 to 24°.
†Reaction rate could not be determined because the method used depended on aceto-CoA-kinase, which was limiting.

TABLE III

Measurement of products of the aconitase and isocitric dehydrogenase reactions

Compound added	Compound formed, μM	
	TPNH	α-Ketoglutarate
Isocitrate	0.6	0.79
cis-Aconitate	0.6	0.80
Citrate	0.3	0.37

NOTE: The complete system contained 100 μM of potassium phosphate, pH 7.5, 10 μM of $MgCl_2$, 0.05 ml of cell-free extract from acetate-grown cells (2 mg of protein), 0.6 μM of TPN, 10 μM of the compound added, and water to 3.0 ml in a Beckman corex cell. TPNH formation was followed at 340 mμ, and the reaction stopped by the addition of 0.3 ml of 100% trichloroacetic acid, and the α-ketoglutarate formed measured by the methods described in the text.

mixtures were treated with 2,4-dinitrophenylhydrazine and chromatographed with known standards. Glyoxylate was identified as a product of the isocitrate splitting reaction in the incubation mixture of extracts from acetate-grown cells, but could not be found in similar mixtures containing extracts of glucose-grown cells.

The activities of malate synthetase and condensing enzymes in Table II were calculated from the data in Table I. The methods used to determine the activities of the other enzymes in Table II are discussed in the section on Materials and Methods.

Discussion

The demonstration of the individual enzymes of the tricarboxylic acid cycle in cell-free extracts of *X. phaseoli*, together with previous work which indicated that such extracts can oxidize intermediates of the cycle (4), suggests strongly that the tricarboxylic acid cycle is the major, if not the only, pathway of terminal oxidation of carbohydrates in this organism. Of the two enzymes necessary to modify the tricarboxylic acid cycle into the glyoxylate cycle, one, isocitritase, appears to be absent entirely in extracts of the organism grown on glucose, while the other, malate synthetase, is present in very small amounts. It is apparent, then, that the organism produces these two enzymes when grown in a medium containing acetate as the sole source of carbon.

X. phaseoli grows much more slowly in the acetate medium than in the glucose – yeast extract medium (mean generation time of 8 to 10 hours instead of 2 to 3), and the levels of enzyme activities recorded in Table II are of the right order to allow the organism to synthesize its cell material from acetate through the mechanism of the glyoxylate cycle.

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THE GROWTH OF UNICELLULAR ALGAE IN ARTIFICIAL AND ENRICHED SEA WATER MEDIA[1]

JACK MCLACHLAN[2]

Abstract

The growth of pure cultures of *Dunaliella euchlora* strains WHOI-1 and WHOI-2, *Platymonas subcordiformis*, *Phaeodactylum tricornutum*, *Chlorella vulgaris*, and *Cyclotella meneghiniana* was compared in two enriched sea water media and two artificial marine media. Three general patterns of growth were observed; the green flagellates grew the same in all media, *Phaeodactylum* and *Chlorella* grew more slowly in the artificial media, and *Cyclotella* did not grow in the artificial medium without organic supplements. The addition of vitamin B_{12} to the artificial medium promoted good growth of *Cyclotella*, and of the species studied this alga was the only auxotroph. Growth in the enriched sea water medium was not affected by a precipitate, but removal of the precipitate markedly affected the rate of growth and the total number of cells obtainable.

Introduction

Although unicellular marine algae have been grown in culture for many years, very little is known of the nutrient requirements of these organisms. In most studies, the diatom *Phaeodactylum tricornutum* (*Nitzschia closterium* f. *minutissima*) has been used as the experimental organism, although this alga is apparently not a normal component of the plankton (10), and most of the other organisms in culture are also "laboratory plankters."

Enriched sea water has been employed as a basal medium in most studies, though it has often been difficult to achieve consistent results (13). This inconsistency has been partly overcome by the use of "aged" sea water (17, 18), although this procedure has not been entirely successful (5). The formation of precipitates has presented another difficulty. By carefully adding nutrients to sea water after it has been autoclaved, cooled, and allowed to come to equilibrium with the carbon dioxide of the air, precipitates can often be avoided (17). This procedure is, however, tedious and increases the chances of introducing contaminants into the culture.

In a few investigations, artificial media have been used (e.g. 2, 15), but in none of these studies has the growth obtained in artificial media been compared with that in enriched sea water. However, Lewin (12) obtained comparable growth of a thallose green alga in enriched sea water and in an artificial medium. Provasoli *et al.* (13) recently listed a variety of artificial marine media, but did not indicate the range of organisms it was possible to grow in these media, nor did they give quantitative data on relative growth rates. It was the purpose of this study to compare the growth of several organisms in enriched sea water and in an artificial medium.

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Contribution No. 993 from the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, U.S.A.
[2]Public Health Service Research Fellow of the National Institutes of Health.

Materials and Methods

In this study four media were used, of which two were synthetic and two were based on natural sea water. The synthetic media consisted of modifications of the ASP medium of Provasoli *et al.* (13). These four media were: (1) modified ASP medium (Table I), (2) modified ASP medium plus 5 ml soil extract per liter, (3) enriched sea water, (4) enriched sea water plus 5 ml soil extract per liter. The artificial media had an initial pH of about 6.5, and that of the sea water media was about 8.0. The water used in the ASP media was prepared according to the procedure of Provasoli and Pintner (14), and the soil extract according to that of Gross (6). The sea water media were enriched by the addition of potassium nitrate, potassium monohydrogen phosphate, sodium silicate, and micronutrients, with the exception of iron, in the concentrations employed in the ASP medium. Iron was added as ferric citrate stabilized with an equal amount of citric acid (by weight) at a concentration of 9.0 micromoles. A relatively high concentration of iron was used as Goldberg (4) found that 80 to 90% of the iron added in this form was adsorbed to the walls of the culture vessel within 1 day.

TABLE I

Composition of the modified ASP medium†

Compound	Concentration in micromoles
$NaCl$	410×10^3
$MgSO_4.7H_2O$	24×10^3
$MgCl_2.6H_2O$	22×10^3
$CaCl_2.2H_2O$	10×10^3
KNO_3	1000
K_2HPO_4	100
$Na_2SiO_3.9H_2O$	100
$FeCl_3.6H_2O$	1.5
H_3BO_3	185
$MnCl_2.4H_2O$	7.0
$ZnCl_2$	0.8
$CoCl_2.6H_2O$	0.02
$CuCl_2.2H_2O$	0.0002
Na_2EDTA*	30

*Sodium ethylenediaminetetraacetate.
†The modified ASP medium differs from the original ASP medium in the omission of the vitamin mix 8, B_{12}, and KCl (*c*, 8000 micromoles).

Pure cultures of the following organisms were used: *Phaeodactylum tricornutum* Bohlin, *Cyclotella meneghiniana* Kützing, *Chlorella vulgaris* (?) Beijerinck, *Platymonas subcordiformis* (Wille) Hazen, and *Dunaliella euchlora* Lerche strains WHOI-1 and WHOI-2. *Cyclotella* and *Phaeodactylum* (10) are diatoms; the others are chlorophycean algae. Stock cultures were maintained in the ASP medium without the addition of soil extract with the exception of *Cyclotella* which was cultured in enriched sea water. The absence of contaminant bacteria was established by the procedure of Spencer (16).

The cultures were grown in 125-ml Erlenmeyer flasks containing 50 ml of medium at a temperature of 16° C. A light intensity of approximately

3000 meter-candles was provided by 40-watt fluorescent lights. The flasks were not shaken or aerated. Growth rates were determined by periodic harvests by cell counts, using a Levy hemocytometer. Eight replicates of the cell counts were made, and the weighted standard error of the mean was 5.0. The results are plotted using the expression $\log_2 N_t/N_0$, where N_0 represents the initial concentration of cells and N_t the concentration at the time of harvest. An increase of one unit on the ordinate corresponds to one division of the population.

Results and Discussion

The results show three general patterns of growth. In the two strains of *Dunaliella euchlora* (Fig. 1) and *Platymonas subcordiformis* (Fig. 2), the growth rates in all four media were similar. However, the growth rates of the two strains of *D. euchlora* were quite different. Strain WHOI-1 grew almost twice as rapidly as strain WHOI-2, though morphologically these two strains are indistinguishable. Moreover, WHOI-2 tended to adhere to the surface of the culture vessels, and to settle out of suspension after active growth had ceased, whereas WHOI-1 remained evenly suspended throughout the medium. There was no indication that any of these organisms are auxotrophic. Gibor (3) likewise found *D. viridis*, *D. salina*, and *Platymonas* sp. to require no growth factors, and Droop (1) stated that *Dunaliella* spp. did not require vitamin B_{12}.

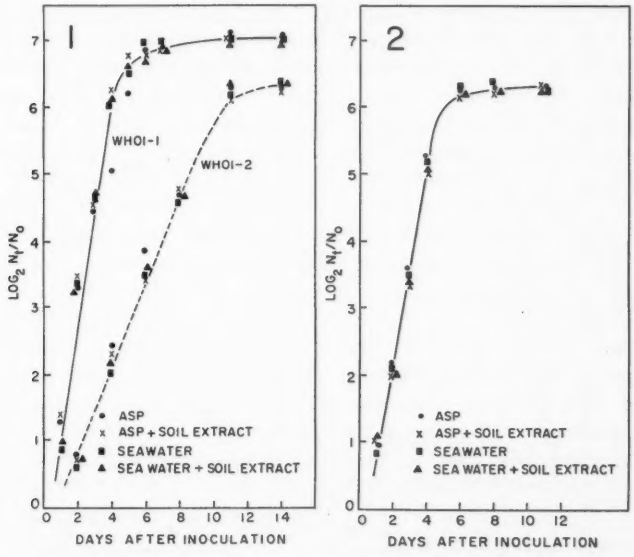

FIG. 1. The growth of *Dunaliella euchlora* strains WHOI-1 and WHOI-2 in the four media.

FIG. 2. The growth of *Platymonas subcordiformis* in the four media.

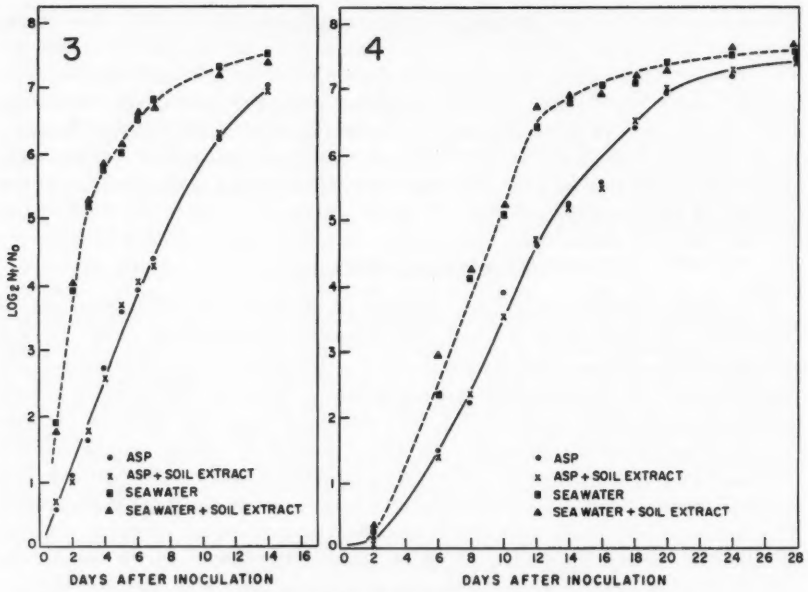

FIG. 3. The growth of *Phaeodactylum tricornutum* in the four media.
FIG. 4. The growth of *Chlorella vulgaris* in the four media.

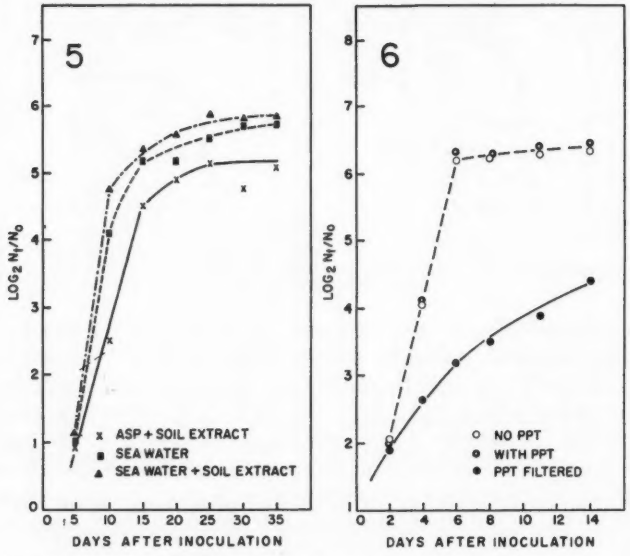

FIG. 5. The growth of *Cyclotella meneghiniana* in the four media.
FIG. 6. The growth of *Dunaliella euchlora* WHOI-1 in the enriched sea water medium without precipitate, with a precipitate, and with the precipitate removed.

Phaeodactylum tricornutum (Fig. 3) and *Chlorella vulgaris* (Fig. 4) both showed a lag phase when cultured in the artificial media, though terminal growth in all series was approximately the same, and soil extract did not prove to be stimulatory. The retardation of growth in the synthetic media may have been due to the low initial pH, but insufficient data preclude any conclusions as to pH effects.

Cyclotella meneghiniana (Fig. 5) grew well in the enriched sea water series and rather more slowly in the ASP – soil extract medium. Here, too, the low initial pH may possibly account for the slower growth in the ASP – soil extract medium. In the ASP medium without soil extract there was no growth. The lack of growth in this medium indicated a possible vitamin requirement. Dr. J. C. Lewin recently completed a vitamin-requirement survey of a number of diatoms. As *Cyclotella meneghiniana* was found to require vitamin B_{12},* it has now proved possible to obtain satisfactory growth of this alga in the ASP medium supplemented with 0.20 μg per liter of vitamin B_{12}. These results demonstrated either that vitamin B_{12} was originally present in the sea water used, or that during the "aging" process, bacterial activities resulted in its accumulation in the water.

It was also found possible to grow *Nannochloris atomus*, *Melosira* sp., and *Skeletonema costatum*, which was contaminated, satisfactorily in the ASP medium without soil extract.

Provasoli *et al.* (13) indicated that the principal objection to most marine media hitherto employed was that they tended to form precipitates. In their work the ASP medium, adjusted to an initial pH of about 7.5, occasionally formed a precipitate after autoclaving. However, this difficulty was not experienced in the present study, possibly because of the lower initial pH.

An experiment was performed to determine if precipitates in the sea water medium affect growth. In one series, nutrients were added aseptically to the medium after it had been autoclaved and cooled; no precipitate was formed. A second series was autoclaved with the nutrients, and a precipitate was formed. The medium from half the flasks in this series was filtered through a VC (100 mμ) "Millipore" filter, and both sets of flasks were re-autoclaved and all series inoculated with *Dunaliella euchlora* WHOI-1. The results (Fig. 6) show that the removal of the precipitate reduced both the rate of growth and the final number of cells. It is evident from this experiment that there is a rapid resolution of salts from the initially formed precipitate, or at least enough so that the rate of growth is not affected. It would also appear that precipitates are not really objectionable; however, as has been pointed out (13), it is difficult to reproduce the same kind of precipitate and, in consequence, to repeat the results of an experiment. Also, if growth is to be measured by optical density or packed cell volume, the objection to precipitates is obvious.

The artificial media used in this study was formulated to resemble sea water with a salinity, based on the weight of the salts, of 35‰. Using data

*Lewin, J. C. Personal communication.

presented by Harvey (7), a comparison of the conservative elements in sea water, with a similar salinity, is presented in Table II. The artificial medium had a slightly lower molarity than sea water and a reduced potassium concentration. Since no carbonate was added, this medium had a relatively low pH and was poorly buffered.

TABLE II

A comparison of the concentration (in micromoles) of the conservative elements in sea water and in the ASP medium

Element	Sea water	ASP
Sodium	482,628	410,000
Potassium	9,976	1,200
Magnesium	54,690	46,000
Calcium	10,479	10,000
Chloride	558,360	442,000
Sulphur (as sulphate)	28,695	24,000

NOTE: The Mg/Ca ratio in sea water is 5.22 and in the ASP medium 4.60, and the monovalent cation/divalent cation ratio in sea water is 7.56 and in the ASP medium 7.34.

In the sea, the ratio of nitrogen to phosphorus is about 20:1, though the total concentration of these elements varies (9), and analyses have shown that the ratio of these elements in phytoplankton is also about 20:1 (7). Since, in the present study, nitrogen and phosphorus were added at a ratio of 10:1, the media contained relatively too much phosphorus. By reducing the concentration of phosphorus and establishing a N/P ratio of 20:1, neither the growth rate nor the total population was affected.* McLachlan and Yentsch,* using *Dunaliella euchlora* WHOI-1, found that at the end of the log phase of growth, all the nitrogen added to the ASP medium had been organically incorporated. Therefore, the limiting factor to growth in the media used appeared to be nitrogen. Ryther (15) stated that certain green algae grew better at a N/P ratio of 5:1 than at a ratio of 15:1, but this does not seem to be true of *D. euchlora* WHOI-1.* Ryther found, however, that a change in the N/P ratio did not affect the growth of *Phaeodactylum*.

Silicate was always added to the medium, though it was only necessary for the diatom *Cyclotella*. Apparently enough silicate goes into solution from the glass vessels to satisfy the requirements of *Phaeodactylum* (11, 17).

Harvey's data (7) show that the concentration of carbon in sea water is approximately 2000 micromoles, though not all of this element is available for metabolic purposes. Since the average C/N ratio in phytoplankton is about 7.5/1 (8), at least an additional 5000 micromoles of carbon would be needed to metabolize all the available nitrogen in the enriched sea water media. As nitrogen has been found to be the limiting factor in these media, a considerable amount of carbon had to enter the medium by diffusion. Spencer (17) stated that the rate of diffusion of carbon dioxide into a sea water medium was sufficiently slow to cause the supply of carbon to become limiting. On the other hand, Provasoli *et al.* (13) claim that carbon dioxide is avidly absorbed

*McLachlan, J. and Yentsch, C. Unpublished.

at pH 8.0 to 8.4, and thus provides a means of supplying carbon without the necessity of shaking or bubbling. The data presented in these experiments tend to support the latter view as the growth rate of *D. euchlora* was not affected until the supply of nitrogen in the medium had become exhausted. It is assumed that the rate of growth of the other species was also arrested by a limitation of the nitrogen supply. Therefore, carbon does not seem to be a limiting factor under the conditions employed in this study.

This study has demonstrated that some marine phytoplankters can be successfully grown in chemically defined media without precipitates. This is of considerable advantage since sea water is a complex and variable medium and not readily available inland. The results of this study also indicate that it would be possible to grow successfully a wide variety of marine organisms in an artificial solution such as the ASP medium.

Acknowledgments

The author wishes to thank Dr. E. M. Hulburt and Dr. R. A. Lewin for identifying the organisms used in this study. Dr. B. H. Ketchum, Dr. R. A. Lewin, and Dr. J. H. Ryther have read the manuscript and offered valuable suggestions.

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NUCLEIC ACID SYNTHESIS BY A LYSOGENIC STRAIN OF ESCHERICHIA COLI INFECTED WITH AN *r*II MUTANT OF COLIPHAGE T2[1]

J. F. WHITFIELD AND K. M. BAIRD

Abstract

Mutants of phage T2, of the group called *r*II mutants, are known to lack the parent phage's ability to form plaques on strains of *Escherichia coli* lysogenic for phage λ. That they are capable of initiating desoxyribonucleic acid synthesis in the infected lysogenic host cells but are incapable of maintaining it has been shown by the present study using T2r_7 and *E. coli* C112 (λ*s*). In contrast with the superficially similar behavior shown by some other phages (e.g. λ*vlh*), ultraviolet irradiation and consequent induction of the lysogenic culture do not release the prophage-linked inhibition of T2r_7 DNA synthesis. Net ribosenucleic acid synthesis is stopped in all cultures on infection with T2r_7.

Introduction

The *r* mutants of coliphages T2, T4, and T6 can be separated into groups on the basis of their action on bacterial strains other than the generally used *Escherichia coli* B. Mutants of the *r*II group are distinguished from other *r* mutants and wild type (r^+) phage by their inability to form plaques on strains of *E. coli* lysogenic for phage λ (2). Such mutants adsorb onto cells of these strains and kill them. Penetration of the viral genetic material into the host cell must occur, since mixed infection with two different *r*II mutants can result in recombination to form wild type recombinants which can multiply normally (2).

The inability of a particular *r*II mutant to form plaques on a lambda lysogen may be a consequence of one or a combination of three types of defect.

(1) The infecting phage may be incapable of initiating the synthesis of its desoxyribonucleic acid (DNA). Such a defect has been observed in a strain of temperate *Bacillus megaterium* phage (14).

(2) The infecting phage may be able to initiate DNA synthesis but unable to maintain it.

(3) There may be a defect in the series of reactions which normally confer upon intracellular vegetative phages (9) the property of infectivity by enclosing them in protein membranes (maturation). This type of defect has been proposed to account for the rarity with which infective particles are liberated from bacteria lysogenic for some strains of λ (7, 16).

To discriminate between these alternatives, we have followed the changes in cellular DNA and RNA content in irradiated and nonirradiated cultures of *E. coli* C112 and *E. coli* C112 (λ*s*) which have been infected with T2r_7, one of a series of *r*II mutants of T2 (6). Our observations show that the

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inability of this mutant to grow on C112 (λs) is associated with the second of the three categories of defect, i.e. the infecting phage can initiate but cannot maintain DNA synthesis in its host cell.

Materials and Methods

The bacterial strains used were *E. coli* strain B, *E. coli* C112, and *E. coli* C112 (λs). The origins of the latter two strains have been described previously (14). *E. coli* C112 (λs) is a lysogenic strain which has the ability to release phage λs following exposure to small doses of ultraviolet light. Phage λs is distinguished by its ability to form small, turbid plaques when plated on *E. coli* C112.

Coliphage T2r_7 was obtained from Dr. A. D. Hershey (Cold Spring Harbor, Long Island, New York).

All phage and bacterial strains were grown and maintained on a tryptone–salts medium (Bacto tryptone, 10 g; NaCl, 5 g; $MgSO_4.7H_2O$, 2.5 g; $CaCl_2$, 0.01 g; $FeCl_3$, 0.0003 g; thiamine hydrochloride, 0.00013 g; agar (optional), 15 g; distilled water, 1000 ml).

For irradiation and infection, cultures of *E. coli* C112 or C112 (λs) were grown overnight at 37° C in 20 ml aerated tryptone broth. Two milliliters of saturated culture were placed into 398 ml fresh broth in an Erlenmeyer flask and the culture was incubated at 37° C with aeration until its turbidity attained a value, nephelometrically determined, corresponding to 3×10^8 cells/ml.

The entire culture was divided into eight lots of 50 ml each and the cells sedimented at 8000 *g* for 5 minutes. The pellets were washed once with phosphate buffer, pooled, and resuspended in 356 to 360 ml buffer (containing the same amounts of Mg, Ca, Fe, and thiamine as the growth medium).

The buffer suspension was poured onto a large, fiberglass–plastic tray (3860 cm^2 area) 75 cm above which was a 15-watt General Electric germicidal lamp. All cultures were irradiated for 50 seconds, a dose sufficient to kill 40 to 50% of the cells in a nonlysogenic culture of C112 or to induce phage development in 95 to 99% of the cells in a culture of C112 (λs). The efficiency of this method of inducing phage development in such large quantities of lysogenic cultures may be judged by the rapidity of lysis between 75 and 80 minutes later (Fig. 7, B). The same manipulations including a 50-second sojourn in the large tray were employed with cultures which were not to be irradiated.

If the culture was to be infected with T2r_7, 2 to 4 ml of phage stock containing no less than 2×10^{11} phage/ml were added to 356 to 358 ml buffer culture. Addition to 40 ml of a 10% (w/v) solution of tryptone in distilled water made up the culture volume to 400 ml and completed the growth medium.

The concentration of viable cells was assayed just prior to irradiation or addition of phage. The phage input was assayed by the agar layer technique immediately after addition of phage and the unadsorbed phage was assayed 7 to 10 minutes after infection.

Determination of Nucleic Acids

Forty-milliliter samples were removed from the culture at appropriate time intervals, pipetted into 50 ml cellulose acetate centrifuge tubes, and rapidly cooled to 6° C in a dry ice – ethanol bath. The cells were sedimented by centrifugation at 8000 *g* for 5 minutes (at 4° C). The pellets were washed with buffer and resuspended in 5 ml 5% trichloroacetic acid (TCA). The acid-soluble nucleotides were extracted at 4° C for 30 minutes in 5% TCA. The cells were sedimented and the pellet resuspended in 2.5 ml 5% TCA and the nucleic acids extracted at 90° C (11, 13).

The concentration of cellular DNA in the hot TCA extract was estimated on the basis of desoxyribose by the diphenylamine method of Dische (5) and the concentration of cellular ribosenucleic acid (RNA) on the basis of its ribose by the orcinol method (10, 11). A highly polymerized sample of calf thymus DNA (obtained from Dr. G. Butler, University of Toronto) and a commercial preparation of yeast RNA (Schwarz Laboratories, New York) served as standards.

Results

Infections of E. coli C112

T2r_7 was able to adsorb onto C112 (about 90% in 7 to 10 minutes). However, it produced fewer plaques when plated on this strain than it did on *E. coli* B (Table I). Streisinger and Weigle (15) noted that the plating efficiency of wild type T2 on *E. coli* 12-112 was low when compared with that on *E. coli* B.

TABLE I

A comparison of the ability of T2r_7 to form plaques on *E. coli* C112, C112 (λs), and *E. coli* B

T2r_7 plated on strain	No. of plaques
B	502
C112	189
C112 (λs)	0

NOTE: A stock of T2r_7 containing 2.51×10^{11} viable phage per ml was diluted 10^{-8} in tryptone broth and 0.1-ml samples were mixed with 2 ml soft agar at 45° C containing cells from saturated cultures of *E. coli* B, *E. coli* C112, or C112 (λs) and were plated. The numbers are sums of the numbers of plaques on two plates.

Following infection of an irradiated or nonirradiated culture there was a lag in net DNA synthesis, which lasted 5 to 10 minutes. After this, the DNA content increased linearly with respect to time for 45 minutes, the total increase in this period being 5 to 6 times the initial content. The rate of DNA increase in irradiated cultures was slightly lower than in nonirradiated cultures. These changes are illustrated by the curves in Fig. 1.

It should be noted that in uninfected cultures of *E. coli* C112, ultraviolet irradiation caused a lag in DNA increase, which lasted between 30 and 40 minutes, after which DNA increase resumed but was *linear* with respect to time (Fig. 2). T2r_7 was able to overcome the ultraviolet-induced lag in DNA synthesis and initiate synthesis of its own DNA.

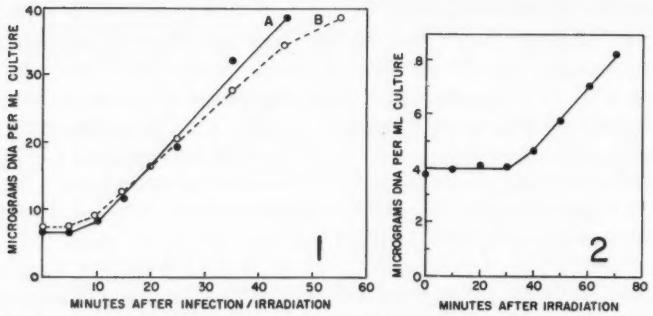

FIG. 1. Changes in the cellular DNA content of irradiated and nonirradiated cultures of *E. coli* C112 infected with T2r_7.

(A) ●———● Nonirradiated culture of *E. coli* C112 containing 3.28×10^8 cells/ml was infected with 2.39×10^9 phage/ml.

(B) ○-----○ An irradiated culture of *E. coli* C112 containing 3.83×10^8 cells/ml was infected with 1.92×10^9 phage/ml.

FIG. 2. Changes in the cellular DNA content of a culture of *E. coli* C112 following ultraviolet irradiation. A culture of C112 containing 2.44×10^8 cells/ml was irradiated. The dose delivered to the culture reduced the titer of viable cells to 1.03×10^8/ml (assayed 40 minutes postirradiation).

As would be expected from the observations of Cohen (3, 4) on cultures of *E. coli* B infected with T2r^+, T2r_7 infection extinguished further change in the RNA content of cultures of C112 for at least 45 minutes.

The turbidity of these cultures decreased (30%) during the first 10 minutes after infection, but between 10 and 55 minutes rose slightly to reach a constant value, after which there was no further change (Fig. 3, A). In one experiment, in which the turbidity was followed for 3.25 hours, there was no change between 65 and 195 minutes after infection. We conclude that lysis was inhibited, or at least much delayed, in these cultures.

Infections of E. coli C112 (λs)

Following infection of nonirradiated *E. coli* C112 (λs) there was either no increase in the DNA content of the culture or only a slow rise for the first 5 to

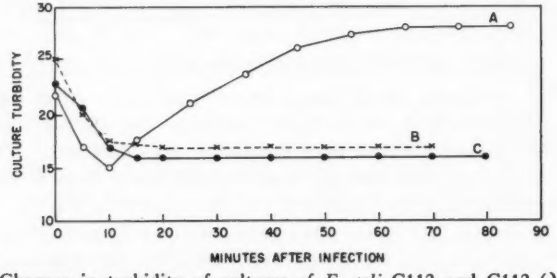

FIG. 3. Changes in turbidity of cultures of *E. coli* C112 and C112 (λs) following infection with T2r_7.

(A) ○———○ *E. coli* C112.

(B) ×------× *E. coli* C112 (λs), nonirradiated.

(C) ●———● *E. coli* C112 (λs), irradiated.

8 minutes, followed by a more rapid increase until 15 minutes. Thereafter, a slow decline in DNA content of the culture was the rule. Although the total increase in DNA was, on the average, very small (1.3 times the initial content), it was observed in all seven experiments. These changes are illustrated in Fig. 4, A.

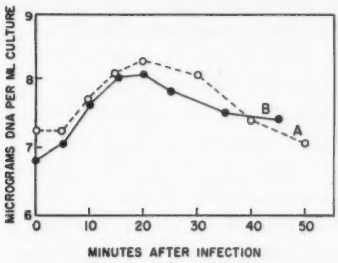

FIG. 4. Changes in cellular DNA content of irradiated and nonirradiated cultures of *E. coli* (λs) infected with T2r_7.

(A) ○------○ A culture of *E. coli* C112 (λs) containing 3.41×10^8 cells/ml was infected with 1.48×10^9 phage/ml; 90% of the phage were adsorbed by 10 minutes. No T2r_7 yielding cells were observed among 700 cells plated on *E. coli* B.

(B) ●———● A culture of C112 (λs) containing 3.14×10^8 cells/ml infected immediately after irradiation with 1.60×10^9 phage/ml; 94% of the phage were adsorbed 15 minutes after infection. No T2r_7 yielders were observed among 628 cells plated on *E. coli* B.

The RNA content of such cultures remained constant following infection. Therefore, T2r_7 extinguishes net RNA synthesis in a strain lysogenic for λ.

As with infected cultures of *E. coli* C112, no lysis was detected in infected cultures of C112 (λs). However, there was no increase in the turbidity of the culture following the initial decrease as there was with infected C112. The turbidity decreased (30%) between 0 and 15 minutes after infection and reached a constant value. This value was maintained for at least 70 minutes (Fig. 3, B).

Preliminary experiments have been carried out to determine the changes in the nuclear structures of *E. coli* C112 (λs) infected with T2r_7. The chromatin was stained according to the method of Whitfield and Murray (17). During the first 5 minutes postinfection the nuclear changes were identical with those in *E. coli* B infected with T2 (8, 12); the host chromatin dispersed and came to lie along the cell boundaries. Between 5 and 15 minutes diffuse chromatin appeared in the central region of the cell. By 15 minutes at least 90% of the cells had diffuse chromatin. After 30 minutes the central busophilism began to fade. These observations clearly indicate that the slight rise in cellular DNA content of C112 (λs) cultures after infection was not due to the presence of a small fraction of normal infections, but was a reflection of a homogeneous response to infection of most cells in a culture. In addition, no lysis was observed at any time between 0 and 85 minutes after infection.

Infections of Irradiated E. coli C112 (λs)

Following ultraviolet irradiation, cultures of *E. coli* C112 (λs) are destroyed by lysis, a consequence of induced growth of λs. In addition, such a culture

supports multiplication of the weak virulent phage λ*vlh* to which the nonirradiated culture is entirely resistant (16). This indicates that, at some time after irradiation, prophage-linked (9) immunity to some phages disappears.

Infection with T2r_7 of a culture of *E. coli* C112 (λ*s*) immediately after irradiation resulted in the initiation of DNA synthesis during the lag period. DNA began to increase during the first 5 minutes after infection (Fig. 4, B). However, there was no difference between the DNA changes in these cultures and the nonirradiated ones described above (compare Fig. 4, A and B). The turbidity changes were identical in both cases (compare Fig. 3, A and B). The RNA content did not change for at least 70 minutes after infection.

No cells were observed (<0.15%) in such cultures which yielded T2r_7 or λ*s* or both. Therefore, development of T2r_7, unlike λ*vlh*, may be inhibited throughout the entire postirradiation period in a lysogenic culture, or it may be that the phage cannot remain intact in the host cell long enough to take advantage of a release of inhibition which occurs at some later time in the latent period.

If the latter explanation be correct, then infection of *E. coli* C112 (λ*s*) during the period of DNA increase might be followed by growth of T2r_7. Infection of C112 (λ*s*) at 42 minutes after irradiation was followed by a cessation of DNA increase for 5 minutes. During the next 7 to 8 minutes the DNA level increased to about 1.3 times the initial value and then remained the same. This sequence of changes was identical with that in a culture of C112 (λ*s*) infected immediately after irradiation (compare Fig. 5, A with Fig. 4, B).

The RNA content of an induced lysogenic culture increased after ultraviolet irradiation. However, 2 minutes after infection with T2r_7, (44 minutes after irradiation) this increase stopped and the RNA content remained constant (Fig. 5, B).

The usual increase in the turbidity of a culture of C112 (λ*s*) following irradiation (Fig. 6, A) was brought to a halt upon infection with T2r_7 (Fig. 6, B).

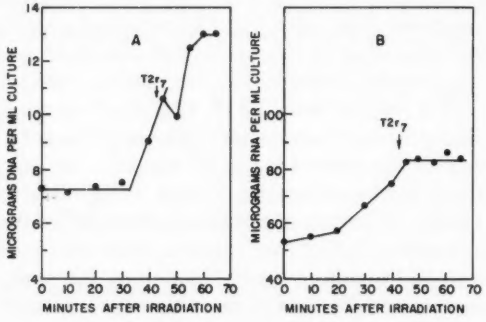

FIG. 5. Changes in the cellular DNA (A) and RNA (B) contents of a culture of irradiated *E. coli* C112 (λ*s*) infected with T2r_7 42 minutes after irradiation. A culture containing 3.48×10^8 cells/ml was infected with 2.06×10^9 T2r_7/ml; 95% of the phage were adsorbed 15 minutes after infection. λ*s* development was induced in 98.3% of the cells but no yielders were observed after T2r_7 infection.

The turbidity dropped (30%) during the first 25 minutes after infection (67 minutes postirradiation) to reach a value which was maintained for at least a further 70 minutes (Fig. 6, B). These changes were the same as those in a culture of C112 (λs) infected immediately after irradiation.

The changes in the nuclear structures in cells from irradiated cultures of *E. coli* C112 (λs) infected with T2r_7 immediately after irradiation were identical with those described above for infected, unirradiated C112 (λs).

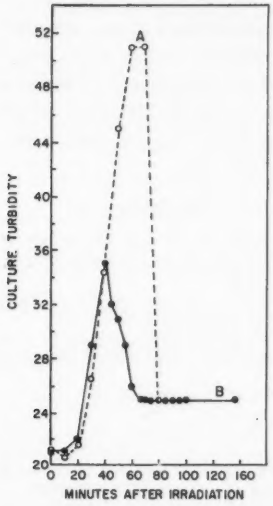

FIG. 6. The effect of T2r_7 infection on turbidity changes in a culture of *E. coli* C112 (λs) infected 42 minutes after irradiation.
(A) ○-----○ Control, irradiated but not infected culture.
(B) ●———● Irradiated and then T2r_7 superinfected culture.

Discussion

From the foregoing observations we may conclude that the inability of T2r_7 to form plaques on strains of *E. coli* lysogenic for coliphage λ (Table I) is associated with a corresponding inability of this phage to maintain net DNA synthesis in such strains. T2r_7 is capable of inititating DNA synthesis, but net synthesis stops shortly after infection when λ prophage is present.

It might have been anticipated that irradiation would release the inhibition for the following reasons. There is no permanent genetic change in the bacterium when it becomes lysogenic, since loss of prophage restores the bacterium's ability to support T2r_7 growth (1). Ultraviolet-induced transition from the prophage to the vegetative state is known to coincide with the resumption of DNA synthesis following irradiation (16), and this transition has been observed to permit superinfecting phage to grow during the postirradiation

latent period in similar experiments with λ*vlh*.* However, in the case of T2r_7 superinfections there was no release of either DNA synthesis or phage production.

We can think either in terms of a requirement or a sensitivity on the part of the infecting phage, which is brought to light only in the lysogenic host. Thus, the mutant r_7 locus might impose upon its bearer a specific requirement for some component essential for normal DNA synthesis; a requirement not exhibited by wild type T2. The λ prophage would then appear as interfering with the postinfection synthesis of this component, an interference which might be associated either with production of an abnormal DNA which is readily broken down, or with production of normal T2r_7 DNA which is limited by the pre-existing supply of the component. Alternatively, T2r_7 might be extraordinarily sensitive to some intracellular factor present in lysogenic cells which intervenes to destroy either T2r_7 or a substance important to its synthesis.

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*Whitfield, J. F. Unpublished.

EFFECT OF SALT CONCENTRATION ON THE MORPHOLOGY AND CHEMICAL COMPOSITION OF MICROCOCCUS HALODENITRIFICANS[1]

I. TAKAHASHI[2] AND N. E. GIBBONS

Abstract

Cells of *Micrococcus halodenitrificans* grown in media containing more than 0.7 *M* sodium chloride appeared normal microscopically, but in 0.6 *M* salt many cells were swollen and in 0.55 *M* salt most cells were swollen or ruptured. The swollen cells were sensitive to osmotic shock. Calcium or magnesium prevented the cells from swelling and extended the lower limit of growth from 0.55 to 0.3 *M* salt. Walls of normal cells contained 6 carbohydrates and 16 amino acids. Qualitative chromatographic analyses indicated that cells grown in 0.55 *M* salt contained less tyrosine, diaminopimelic acid, and an unknown ninhydrin-positive, cytoplasmic component than cells grown in 1.0 *M* salt. Quantitative estimates indicated that diaminopimelic acid/nitrogen ratio in cells decreased gradually from 0.25 to 0.16 as the salt content of the growth medium decreased from 1.0 to 0.55 *M*, but that it decreased to 0.003 in cells grown in 0.3 *M* salt plus calcium. The results suggest that less cell wall material is produced as the salt concentration in the growth medium is decreased and that calcium has a protective effect on the weakened cells or protoplasts.

Introduction

Any explanation of bacterial halophilism must take into consideration not only the salt tolerance but also the salt dependence of the organism. Salt tolerance appears to be linked with the adaptation of the enzymes of halophilic bacteria to high salt concentrations (1) but this only partially explains the minimal requirements of the halophiles for salt.[3] The presence of extracellular nucleic acids (NA) in cultures of *Micrococcus halodenitrificans* and *Vibrio costicolus* grown at low salt concentrations (17) suggested that the permeability of the cells was altered under these conditions. However, it was demonstrated (19) that the extracellular NA in cultures of the micrococcus was intracellular in origin and increased in amount as the salt concentration was reduced from 0.7 to 0.55 *M*, the usual lower limit of growth. These observations, together with the appearance of abnormal cells in cultures grown at low salt concentrations, suggested that salt dependence might be related to cell wall synthesis. To obtain further information, studies were made of the effect of various salts and salt concentrations on the morphology of the cells and cell walls, on the chemical composition of whole cells and cell walls, and on the osmotic properties of the cells.

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Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 4991.
[2]National Research Council Postdoctorate Fellow 1956–58. Present address: Dairy Technology Building, Central Experimental Farm, Ottawa, Ontario.
[3]Throughout this paper salt refers to sodium chloride; other salts will be mentioned specifically.

Methods

Cultural Methods

Growth media contained 0.5% each of proteose–peptone (Difco) and tryptone (Difco) and the required amount of salt. A dairy grade sodium chloride was used (average analysis[4] NaCl 99.75%, $CaSO_4$ 0.23%, $CaCl_2$ 0.01%, $MgCl_2$ 0.01%); other added salts were c.p. grade. A strain of *M. halodenitrificans* was maintained on 1.0 *M* salt agar. Cells were grown in 1.0 *M* salt broth, harvested by centrifugation, washed twice in 1.0 *M* salt solution, suspended in 1.0 *M* salt solution, and sufficient cells (usually 0.2 ml) added to 100-ml portions of broth in 250-ml flasks to give an initial optical density of approximately 0.03 at 660 mμ. Unless otherwise specified, all cultures were incubated at room temperature (20–25° C) on a rotary shaker for 20 hours.

Total cell counts were made microscopically using a Petroff–Hausser slide and viable counts by pour plates using 1.0 *M* salt agar. Optical density measurements were made at 660 mμ using a Coleman Junior spectrophotometer.

Microscopical Examination

Wet preparations were examined with a Spencer phase-contrast microscope and photomicrographs taken with a Spencer camera using Kodak Super Pancro-Press type B film.

Specimens for electron micrographs were dried on collodion films supported on copper grids and fixed in the vapor of 2% osmium tetroxide for 3 to 5 minutes. Salts were then removed from the samples by the addition and removal of several drops of distilled water.

Preparation of Cell Walls

When cells were shaken in distilled water in the Mickle disintegrator with Ballotini beads (No. 12) as described by Salton (14), the cell walls fragmented. This was prevented to some extent by shaking in 1.0 *M* salt solutions but as the yields were still poor, the following procedure was adopted for some preparations. Whole cells, suspended in the appropriate salt solution, were heated in a water bath at 85° C for 5 minutes. The suspensions became very viscous and the cells were dispersed by treatment with deoxyribonuclease (DNase) at room temperature for 30 minutes. The cells were then washed, suspended in distilled water, and shaken in the Mickle disintegrator for 15 minutes. These cell walls, referred to as heated, were then purified as described by Salton (14).

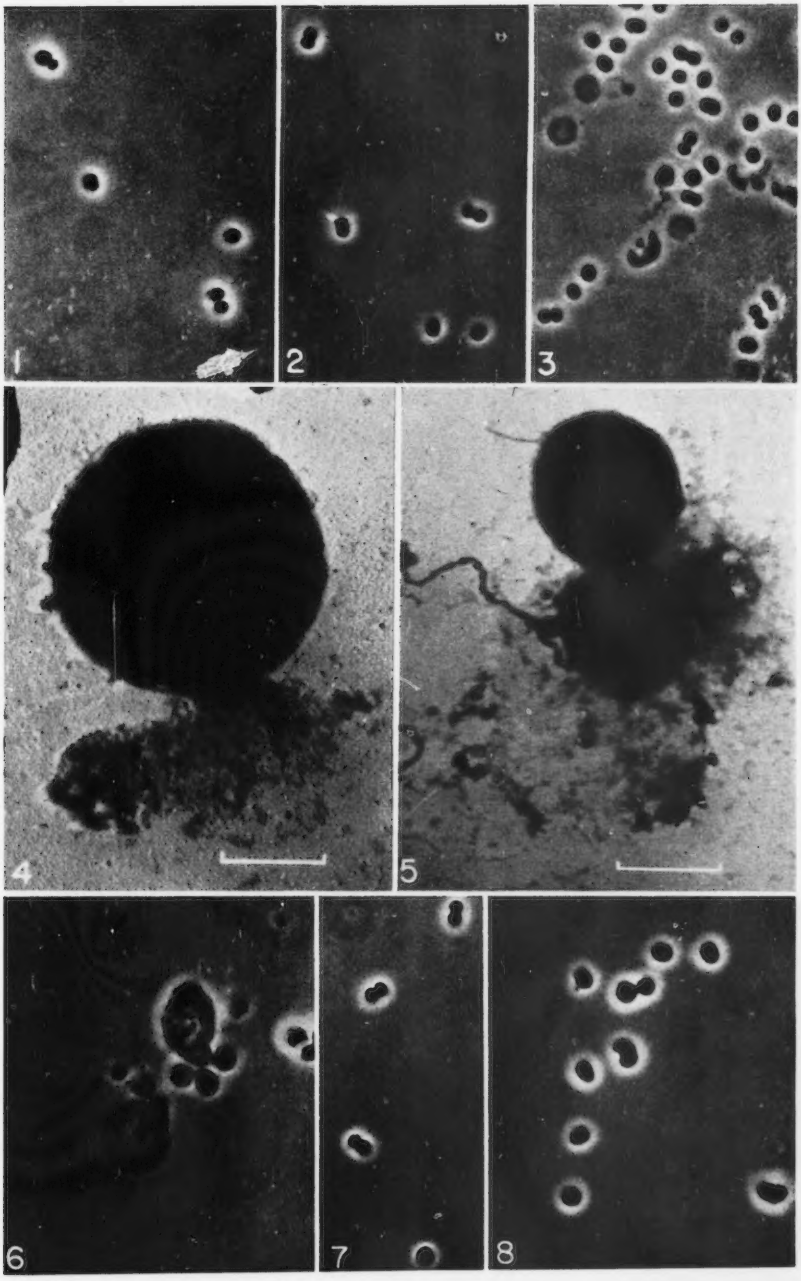
[4]Supplied by Canadian Salt Company, Windsor, Ontario.

FIGS. 1 to 3 and 6 to 12. Phase-contrast photomicrographs of *M. halodenitrificans*. Magnification, × 1540.

FIGS. 4 and 5. Electron photomicrographs. Scale 1 μ.

Fig. 1. Cells grown in 1 *M* NaCl; FIG. 2, in 0.7 *M* NaCl; FIGS. 3, 4, 5, in 0.6 *M* NaCl; FIG. 6, in 0.55 *M* NaCl; FIG. 7, in 0.6 *M* NaCl + 0.005 *M* $CaCl_2$; FIG. 8, in 0.3 *M* NaCl + 0.005 *M* $CaCl_2$; FIG. 9, in 0.6 *M* NaCl + 0.1 *M* KCl; FIG. 10, in 0.6 *M* NaCl + 0.3 *M* KCl; FIG. 11, in 0.4 *M* NaCl + 0.3 *M* KCl; FIG. 12, cells added to 0.4 *M* NaCl + 0.58 *M* sucrose.

PLATE I



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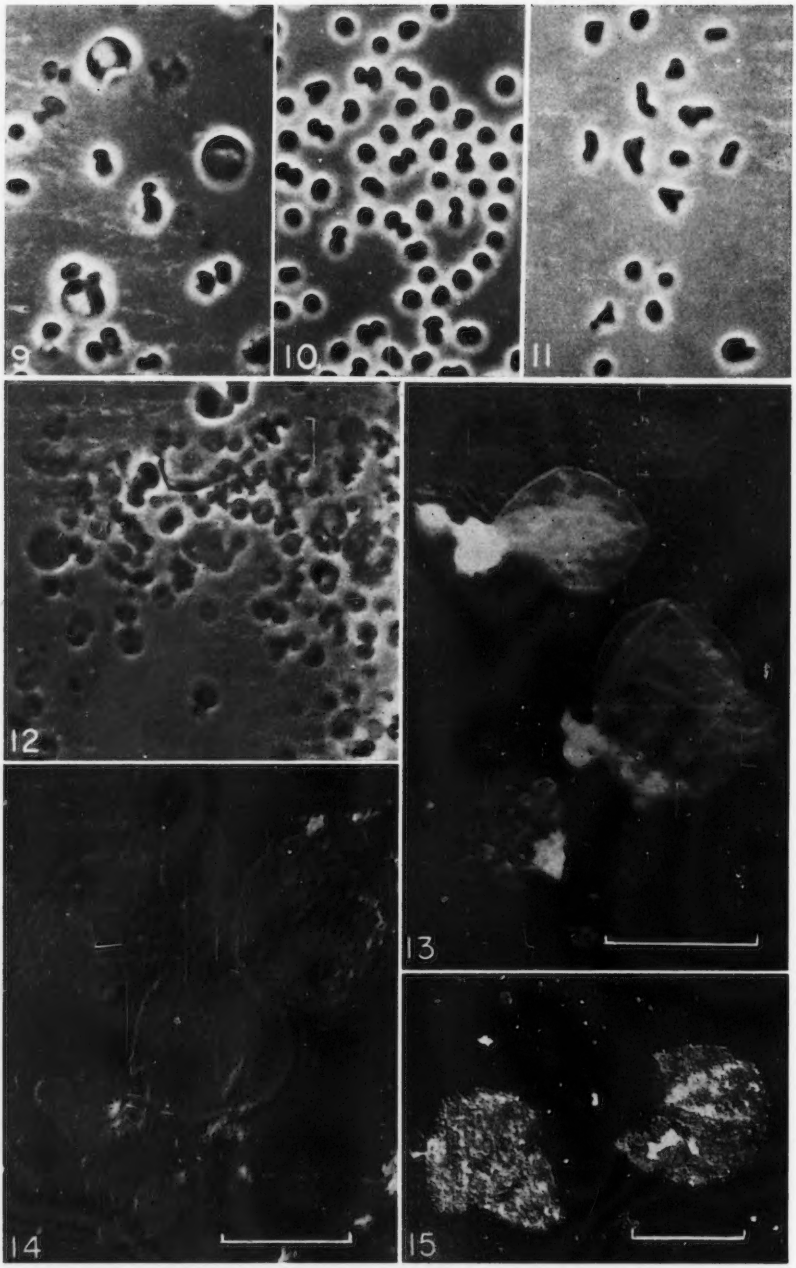

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Chromatographic Methods

For amino acid analysis, samples were hydrolyzed in 6 *N* hydrochloric acid in sealed ampoules at 100° C for 20 hours. Hydrolyzates were filtered, evaporated to dryness on a boiling-water bath, and redissolved in distilled water. This was repeated several times. They were then desalted with a Reco electric desalter,[5] and treated with hydrogen peroxide as described by Dent (4). Two-dimensional ascending chromatograms (33×33 cm Whatman No. 4 filter paper) were prepared using water-saturated phenol in an ammoniacal atmosphere as the first solvent, and *n*-butanol–acetic acid–water (40:10:50) as the second solvent. The chromatograms were sprayed with ninhydrin solution (0.2% in 95% acetone and 5% water) and heated at 100° C for 3 minutes. The presence of α,ϵ-diaminopimelic acid (DAP) was confirmed by one-dimensional chromatography as described by Hoare and Work (6). In some experiments DAP was estimated quantitatively by the colorimetric method of Work (21) and by comparison with known amounts of DAP using a recording integrating densitometer (Spinco Analytrol Model RA) and a No. 98 Kodak Wratten filter.

For sugar analysis, samples were hydrolyzed in 2 *N* sulphuric acid in sealed ampoules at 100° C for 2 hours. Hydrolyzates were neutralized with barium hydroxide, centrifuged, and the supernates evaporated to dryness *in vacuo* over phosphorus pentoxide. The residue was taken up in distilled water and desalted as described above. Duplicate one-dimensional chromatograms were prepared according to the technique described by Martin (9). One was developed with alkaline silver nitrate and the other with *p*-anisidine–HCl.

Other Methods of Analysis

Nitrogen and reducing sugars were determined by methods described previously (16). Phosphorus was determined by the method of King (7); chlorides by the Volhard method.

Results

Effect of Salts on Cell Morphology

In cultures containing more than 0.7 *M* salt, the cells appeared normal (Figs. 1 and 2) and the amount of extracellular NA was negligible. Cultures in 0.6 *M* salt contained considerable amounts of extracellular NA and on microscopic examination normal cells, swollen cells resembling the protoplasts described by other authors (5, 10, 20), and ruptured cells were found (Figs. 3, 4, 5). In 0.55 *M* salt, the lowest salt concentration at which growth occurred when dairy grade salt was used, almost all the cells appeared swollen or ruptured (Fig. 6).

[5]Made by Research Equipment Corporation, Berkeley, California.

FIGS. 13 to 15. Electron photomicrographs of cell walls of *M. halodenitrificans*.
FIG. 13. Cell walls prepared from cells grown in 1 *M* NaCl (unheated); FIG. 14, from cells grown in 1 *M* NaCl (heated); FIG. 15, from cells grown in 0.6 *M* NaCl + 0.3 *M* KCl (heated).

Cultures in 0.6 *M* salt plus 0.005 *M* calcium chloride contained little extracellular NA (19) and the cells appeared normal (Fig. 7). With this same concentration of calcium, the organism could grow in 0.3 *M* salt, although the cells were slightly larger than those grown in 1.0 *M* salt (Fig. 8). Magnesium had about the same effect as calcium. On the other hand, cultures grown in 0.6 *M* sodium chloride plus 0.1 *M* potassium chloride were viscous and contained a large proportion of swollen cells (Fig. 9). When higher concentrations of KCl were used (0.3 *M*) the cultures were not viscous and cells appeared only slightly irregular and somewhat larger than normal cells (Fig. 10). When the sodium chloride concentration was reduced (0.4 *M* NaCl + 0.3 *M* KCl) the cells did not rupture but there was marked pleomorphism (Fig. 11). An approximately isosmotic mixture with sucrose (0.4 *M* NaCl + 0.58 *M* sucrose) did not support growth and cells added to it became swollen (Fig. 12).

Osmotic Properties of Cells

The above observations were made on cells from growing cultures and indicated that the changes observed were not entirely the result of differences in osmotic pressures. However, to obtain some information on the effect of salt concentration on the cell walls, the response of cells to abrupt changes in osmotic pressure was studied.

Cells grown in various concentrations of salt or salts were washed and thick suspensions were made in 1.0 *M* salt solution. Aliquots (0.2 ml) of these

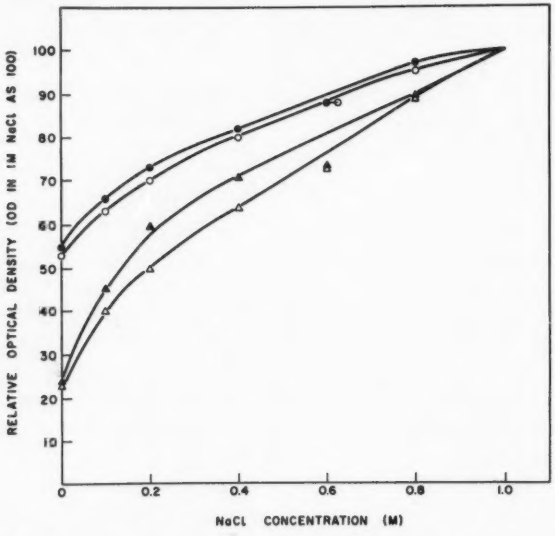

FIG. 16. Effect of salt concentration on the optical density of suspensions of *M. halodenitrificans*. ● Cells grown in 1 *M* NaCl; ○ in 0.6 *M* NaCl + 0.005 *M* $CaCl_2$; ▲ in 0.6 *M* NaCl + 0.3 *M* KCl; △ in 0.3 *M* NaCl + 0.005 *M* $CaCl_2$.

suspensions were then added to 10-ml portions of salt solutions of concentrations lower than 1.0 *M* and buffered with *M*/100 phosphate at pH 7.25. The final suspensions were allowed to stand at room temperature for 20 minutes and the optical density was then measured.

The optical density of suspensions of cells grown in 1:0 *M* salt and of cells grown in 0.6 *M* salt plus calcium decreased gradually as the concentration of salt was decreased (Fig. 16). No sharp decrease in optical density was noted as has been observed with *Vibrio costicolus* and halophilic rods (2). Cells grown in 0.6 and 0.55 *M* salt, in 0.6 *M* salt + 0.3 *M* KCl, or in 0.3 *M* salt + calcium, on the other hand, did lyse at salt concentrations less than 0.2 *M*. In a similar experiment, in which the salt was dissolved in broth instead of water, no marked change in optical density or in total cell count was observed even in the absence of added salt and no marked change in viable count was observed at salt concentrations down to 0.2 *M* (Table I).

TABLE I

Effect of salt concentration on the optical density, total and viable cell count of *M. halodenitrificans**

Salt concentration in medium, *M*	Relative optical density, %	Total cell count/ml, $\times 10^6$	Viable cell count/ml, $\times 10^6$
1.0	100	42	28
0.7	87	38	26
0.6	83	30	22
0.6 + Ca (0.005 *M*)	89	40	21
0.4	79	24	21
0.2	79	26	21
0	74	22	0.0001

*Cells grown 20 hours in medium containing 1.0 *M* salt, suspended in broth containing salt concentrations shown, allowed to stand at room temperature for 2 hours, and optical density, total and viable count determined.

Cells grown in media containing salt concentrations of 0.7 *M* or higher, or in 0.6 *M* salt + calcium, were normal in appearance and the optical density of suspensions in *M*/100 phosphate buffer was about half that of suspensions in 1.0 *M* salt (Table II). However, cells grown in salt concentrations where growth occurred only if calcium or potassium was added appeared to be more fragile than cells grown in molar salt, since the optical density of suspensions in buffer was only about a quarter of that in 1.0 *M* salt. Such cultures contained many swollen cells and it is likely that these were non-viable (Table III) and very susceptible to osmotic shock.

Chemical Composition of Cell Walls in Relation to Morphology

The susceptibility of cells to osmotic shock appeared to be related to the structure of the cell walls. Electron micrographs indicated that walls of cells grown in 1.0 *M* salt (Figs. 13, 14) were rigid and were similar to those of non-halophilic cocci, whereas cells grown in 0.6 *M* NaCl + 0.3 *M* KCl had a thin wall with a rough surface (Fig. 15) and it seemed possible that this morphological difference might be the result of differences in the chemical composition. Walls of cells grown under various conditions were therefore

TABLE II

Susceptibility to osmotic shock of cells of *M. halodenitrificans* grown at various salt concentrations

Concentration of salts in growth medium		Optical density at 660 mμ of cells suspended in:		Decrease in optical density, %
NaCl, *M*	Other salts, *M*	1.0 *M* NaCl	Buffer	
1.0		0.190	0.105	45
0.7		0.230	0.120	48
1.0	$CaCl_2$ 0.005	0.298	0.205	31
0.6	$CaCl_2$ 0.005	0.328	0.175	47
0.4	$CaCl_2$ 0.005	0.150	0.060	60
0.3	$CaCl_2$ 0.005	0.360	0.085	76
0.6	KCl 0.3	0.145	0.033	77
0.4	KCl 0.3	0.100	0.025	75

TABLE III

Effect of salt concentration in the growth medium on viability of *M. halodenitrificans**

Salt concentration in growth medium, *M*	Total cell* count/ml, $\times 10^7$	Viable cell* count/ml, $\times 10^7$	Ratio, viable to total count
2.00	184	168	0.91
1.00	320	250	0.78
0.85	225	138	0.61
0.70	210	128	0.61
0.60	162	75	0.46
0.61 + Ca 0.005 *M*	308	274	0.89
0.55	32	<0.1	<0.003

*Counts made after 20 hours' incubation.

TABLE IV

Amino acid composition of various fractions of *M. halodenitrificans*

Amino acid*	Cell walls,† 1.0 *M*	Cytoplasm, 1.0 *M*	Whole cells: 1.0 *M*‡	Whole cells: 0.6 *M*	Whole cells: 0.55 *M*	Fibrous material, 0.55 *M*
Glycine	++§	+++	++	++	++	++
Lysine	++	+++	++	++	+++	++
Cysteic acid	+	+	+	+	++	+++
Methionine sulphone	+	+	+	+	+	++
DAP	+++	−	+	+‖	−	−
Tyrosine	++	++	++	+	−	−
Unknown	−	++	++	+	−	−

*All fractions contained similar amounts of glutamic acid, aspartic acid, serine, threonine, alanine, valine, leucine/isoleucine, and arginine (+++) and of proline and phenylalanine (++).

†Molarities refer to sodium chloride concentration in growth medium. Unheated and heated cell walls from cells grown in 1.0 *M* NaCl and heated cell walls from cells grown in 0.6 *M* NaCl + 0.3 *M* KCl had the same composition.

‡Whole cells from 1.0 *M* NaCl, 0.6 *M* NaCl + 0.3 *M* KCl, and 0.6 *M* NaCl + 0.005 *M* $CaCl_2$ had the same composition.

§Number of + signs indicates relative size of spots on chromatograms.

‖Presence of DAP confirmed by one-dimensional chromatography.

analyzed for nitrogen, reducing sugars, and total phosphorus. The carbohydrate and amino acid composition of hydrolyzates of cells and cell walls was determined by paper chromatography. It was not possible to obtain sufficient amounts of cell walls of organisms grown in low salt concentrations for analysis and only heated cell walls of organisms grown in 0.6 *M* salt + 0.3 *M* KCl could be obtained. Since no difference could be detected in the composition of heated and unheated walls of cells grown in 1.0 *M* salt, it seemed likely that no difference would be detected in walls of cells grown at other salt concentrations.

Walls of cells grown in 1.0 *M* salt (heated and unheated) and in 0.6 *M* salt + 0.3 *M* KCl (heated) contained 10.5 to 12.0% nitrogen, 2.9 to 3.2% reducing sugars, and 0.61 to 0.73% total phosphorus. There was no difference in the carbohydrate composition of these three samples. Glucose, galactose, and mannose, and lesser amounts of arabinose, xylose, and hexosamine, were present in all samples. The absence of ribose indicated that these cell wall preparations were not contaminated with cytoplasm.

The walls of *M. halodenitrificans* contain at least 16 amino acids, including phenylalanine, tyrosine, cystine/cysteine, methionine, arginine, proline, and DAP (Table IV). The size of the spots indicated that the cell walls of this organism contained a fairly large amount of DAP, which, according to Salton (15), is usually absent from the walls of non-halophilic cocci. The R_f value on one-dimensional chromatograms suggested that the DAP in this organism was the meso- or DD-isomer, but no attempts were made to identify it further. Since the cytoplasm, prepared from cells grown in 1.0 *M* salt and broken in the Mickle disintegrator, did not contain DAP (Table IV), it would appear that this amino acid is present only in the cell walls.

The walls of Gram-positive cocci are characterized by the presence of a limited number of amino acids and the absence of arginine, proline, and the aromatic and sulphur-containing amino acids (15). The number of amino acid constituents, together with the high lipid content reported by Smithies *et al.* (16), suggests that *M. halodenitrificans* has a cell wall similar to Gram-negative bacteria. In fact, this organism was originally described as Gram-negative (13).

Material Resembling Cytoplasmic Membrane

A white fibrous material was noticed in viscous cultures and, although never great, the amount did increase with the viscosity of the culture. This material was soluble in dilute alkali, insoluble in water or acid, and was not solubilized by pancreatic DNase. The ultraviolet absorption curve did not show a peak at 260 mμ but rather resembled that of the cell walls reported previously (16). Two-dimensional chromatograms of hydrolyzates of the fibrous material were characterized by strong spots of sulphur-containing amino acids and the absence of tyrosine and DAP, indicating that it was not formed from disintegrating cell walls (Table IV). The amino acid constituents of the fibrous material were found to be identical qualitatively with those of whole cells grown in 0.55 *M* salt. This material is probably formed,

TABLE II

Susceptibility to osmotic shock of cells of *M. halodenitrificans* grown at various salt concentrations

Concentration of salts in growth medium		Optical density at 660 mμ of cells suspended in:		Decrease in optical density, %
NaCl, *M*	Other salts, *M*	1.0 *M* NaCl	Buffer	
1.0		0.190	0.105	45
0.7		0.230	0.120	48
1.0	$CaCl_2$ 0.005	0.298	0.205	31
0.6	$CaCl_2$ 0.005	0.328	0.175	47
0.4	$CaCl_2$ 0.005	0.150	0.060	60
0.3	$CaCl_2$ 0.005	0.360	0.085	76
0.6	KCl 0.3	0.145	0.033	77
0.4	KCl 0.3	0.100	0.025	75

TABLE III

Effect of salt concentration in the growth medium on viability of *M. halodenitrificans**

Salt concentration in growth medium, *M*	Total cell* count/ml, $\times 10^7$	Viable cell* count/ml, $\times 10^7$	Ratio, viable to total count
2.00	184	168	0.91
1.00	320	250	0.78
0.85	225	138	0.61
0.70	210	128	0.61
0.60	162	75	0.46
0.61 + Ca 0.005 *M*	308	274	0.89
0.55	32	<0.1	<0.003

*Counts made after 20 hours' incubation.

TABLE IV

Amino acid composition of various fractions of *M. halodenitrificans*

Amino acid*	Cell walls,† 1.0 *M*	Cytoplasm, 1.0 *M*	Whole cells: 1.0 *M*‡	Whole cells: 0.6 *M*	Whole cells: 0.55 *M*	Fibrous material, 0.55 *M*
Glycine	++§	+++	++	++	++	++
Lysine	++	+++	++	++	+++	++
Cysteic acid	+	+	+	+	++	+++
Methionine sulphone	+	+	+	+	+	++
DAP	+++	−	+	+‖	−	−
Tyrosine	++	++	++	+	−	−
Unknown	−	++	++	+	−	−

*All fractions contained similar amounts of glutamic acid, aspartic acid, serine, threonine, alanine, valine, leucine/isoleucine, and arginine (+++) and of proline and phenylalanine (++).

†Molarities refer to sodium chloride concentration in growth medium. Unheated and heated cell walls from cells grown in 1.0 *M* NaCl and heated cell walls from cells grown in 0.6 *M* NaCl + 0.3 *M* KCl had the same composition.

‡Whole cells from 1.0 *M* NaCl, 0.6 *M* NaCl + 0.3 *M* KCl, and 0.6 *M* NaCl + 0.005 *M* $CaCl_2$ had the same composition.

§Number of + signs indicates relative size of spots on chromatograms.

‖Presence of DAP confirmed by one-dimensional chromatography.

analyzed for nitrogen, reducing sugars, and total phosphorus. The carbohydrate and amino acid composition of hydrolyzates of cells and cell walls was determined by paper chromatography. It was not possible to obtain sufficient amounts of cell walls of organisms grown in low salt concentrations for analysis and only heated cell walls of organisms grown in 0.6 *M* salt + 0.3 *M* KCl could be obtained. Since no difference could be detected in the composition of heated and unheated walls of cells grown in 1.0 *M* salt, it seemed likely that no difference would be detected in walls of cells grown at other salt concentrations.

Walls of cells grown in 1.0 *M* salt (heated and unheated) and in 0.6 *M* salt + 0.3 *M* KCl (heated) contained 10.5 to 12.0% nitrogen, 2.9 to 3.2% reducing sugars, and 0.61 to 0.73% total phosphorus. There was no difference in the carbohydrate composition of these three samples. Glucose, galactose, and mannose, and lesser amounts of arabinose, xylose, and hexosamine, were present in all samples. The absence of ribose indicated that these cell wall preparations were not contaminated with cytoplasm.

The walls of *M. halodenitrificans* contain at least 16 amino acids, including phenylalanine, tyrosine, cystine/cysteine, methionine, arginine, proline, and DAP (Table IV). The size of the spots indicated that the cell walls of this organism contained a fairly large amount of DAP, which, according to Salton (15), is usually absent from the walls of non-halophilic cocci. The R_f value on one-dimensional chromatograms suggested that the DAP in this organism was the meso- or DD-isomer, but no attempts were made to identify it further. Since the cytoplasm, prepared from cells grown in 1.0 *M* salt and broken in the Mickle disintegrator, did not contain DAP (Table IV), it would appear that this amino acid is present only in the cell walls.

The walls of Gram-positive cocci are characterized by the presence of a limited number of amino acids and the absence of arginine, proline, and the aromatic and sulphur-containing amino acids (15). The number of amino acid constituents, together with the high lipid content reported by Smithies *et al.* (16), suggests that *M. halodenitrificans* has a cell wall similar to Gram-negative bacteria. In fact, this organism was originally described as Gram-negative (13).

Material Resembling Cytoplasmic Membrane

A white fibrous material was noticed in viscous cultures and, although never great, the amount did increase with the viscosity of the culture. This material was soluble in dilute alkali, insoluble in water or acid, and was not solubilized by pancreatic DNase. The ultraviolet absorption curve did not show a peak at 260 mμ but rather resembled that of the cell walls reported previously (16). Two-dimensional chromatograms of hydrolyzates of the fibrous material were characterized by strong spots of sulphur-containing amino acids and the absence of tyrosine and DAP, indicating that it was not formed from disintegrating cell walls (Table IV). The amino acid constituents of the fibrous material were found to be identical qualitatively with those of whole cells grown in 0.55 *M* salt. This material is probably formed,

after rupture of swollen cells, from the cell ghosts which consist mainly of cytoplasmic membrane. However, further study will be necessary to confirm the identity of this material.

Chemical Composition of Whole Cells

Since cell walls of cells grown in low concentrations of salt could not be obtained, whole cells were analyzed. Cells grown in 1.0 *M* salt or in 0.6 *M* salt + 0.3 *M* KCl contained about 8% nitrogen, 2% reducing sugars, and 1% phosphorus. The carbohydrate composition of the cells was not affected by the medium and differed from that of the cell walls only in that ribose was present.

The amino acid composition of hydrolyzates of cells grown in 1.0 *M* salt, in 0.6 *M* salt + 0.3 *M* KCl, and in 0.6 *M* salt + 0.005 *M* $CaCl_2$ was identical (Table IV). However, a change in amino acid composition was noted in cells grown in salt concentrations lower than 0.6 *M*. Chromatograms of non-viscous cells contained tyrosine and an unidentified spot, which had an R_f value in phenol similar to that of glutamic acid, but which ran slightly faster than glutamic acid in *n*-butanol – acetic acid – water. These spots became smaller in cells grown in 0.6 *M* salt and could not be detected in cells grown in 0.55 *M* salt. Tyrosine is present in both cell wall and cytoplasmic material, but the unknown ninhydrin-positive compound is a cytoplasmic component. DAP, a cell wall constituent, could be detected in 0.6 *M* cells only if the sample used was two to three times larger than for 1.0 *M* cells. Whole cells grown in 0.55 *M* salt, therefore, contain very little tyrosine, DAP, and the unknown compound. This suggested that the salt content of the medium might affect the distribution of amino acids in the cells.

The decrease in DAP with decreasing salt concentration might be the result of the destruction of cell walls by lytic enzymes, similar to those described by Strange and Dark (18) and by Nomura and Hosoda (12), which might normally be inhibited by the higher concentrations of salt. If this were so, DAP should be present in the culture supernates. However, DAP could not be detected in hydrolyzates of 5-ml portions of supernates of cultures grown in 1.0, 0.6, and 0.55 *M* salt. Furthermore, no indication of enzyme activity could be detected by changes in optical density when cell suspensions were incubated with cell extracts and culture supernates in phosphate buffer at pH 6 to 8 over a salt range of 0 to 1.0 *M*.

Since cell wall synthesis seemed to depend on the salt concentration of the growth medium, a quantitative estimation was made of the amount of DAP produced at various salt concentrations and in the presence and absence of calcium and magnesium. Since only small amounts of DAP were involved at the low salt concentrations, large amounts of concentrated hydrolyzates had to be used. Usually a drop in the DAP/N ratio could be detected as the amount of salt in the growth medium decreased (Table V). Only one analysis was made with cells grown in 0.54 *M* salt + calcium, and, although the number of cells produced was greater, the ratio of DAP to N was no

greater than in the absence of calcium.[6] There was, however, a definite decrease in the DAP/N ratio in cells from 0.33 *M* salt + calcium or magnesium, and these cells had the same distorted appearance under the microscope as cells from 0.55 *M* salt without added calcium. It would, therefore, seem that in media containing around 0.55 *M* sodium chloride (dairy grade), calcium becomes limiting, and around 0.3 *M* sodium chloride in the presence of 0.005 *M* calcium or 0.01 *M* magnesium, sodium chloride becomes limiting for cell wall synthesis.

TABLE V

Ratio of diaminopimelic acid to total nitrogen in cells of *M. halodenitrificans* grown in media containing different amounts of salt

NaCl, *M*	Other salts, *M*	Wet weight cells/200 ml	DAP/N	
1.05*		5.97	.025†	.033‡
0.62		5.99	.021	.020
0.58		5.16	.016	.011
0.53		2.63	.016	.009
0.54	Ca 0.005	5.66	.014	—
0.33	Ca 0.005	2.64	.002	.005
0.33	Mg 0.01	3.63	.006	.006
0.20	Ca 0.005	No growth		
0.15	Ca 0.005	No growth		

*Determined by chloride analysis.
†DAP measured on chromatograms by densitometer (average of two determinations).
‡DAP measured by method of Work (21).

Discussion

Microscopic examination of cultures of *M. halodenitrificans* revealed that the cells changed from normal coccus forms in media containing 0.7 *M* sodium chloride to swollen and ruptured forms in media containing 0.55 *M* salt. The swollen cells are probably "protoplasts", if this term is used in the sense of McQuillen (11); some cell wall was still present since small amounts of DAP could be detected.[6] The "protoplasts" were very sensitive to osmotic shock and when suspended in distilled water all lysed almost instantly. In cultures growing at low salt concentrations, a white fibrous material, which was lacking in DAP and presumed to be the cytoplasmic membranes of the ruptured cells, was observed. From this and other evidence (19), it may be concluded that the extracellular NA found in cultures of this organism grown at low salt concentrations are from cytoplasmic and nuclear components released from lysed cells.

The "protoplasts" may be formed either through dissolution of cell walls by lytic enzymes or through failure of the organism to synthesize new cell walls during division. Although several cell wall lytic enzymes have been reported recently in *Bacillus* species (12, 18), attempts to demonstrate similar enzymes in cultures of *M. halodenitrificans* were unsuccessful. The possibility of protoplast formation by lysis of the cell walls, therefore, seems

[6]The term "spheroplast" has now been suggested for this type of cell (McQuillen, personal communication).

remote. The fact that the effects of low salt concentrations were observed only in growing cultures suggests that cell wall synthesis is directly concerned. Also the decrease in DAP content with decreasing salt concentration suggested that cell wall synthesis was susceptible to small differences in salt concentration, although the possibility of an ion being limiting was not ruled out entirely. The decrease in tyrosine and the ninhydrin-positive substance in cells grown in low salt concentrations suggested that synthetic mechanisms were affected by changes in salt concentration. It is not known, however, whether these substances are concerned with cell wall synthesis or some other metabolic process. Since it is known that a number of enzymes of halophilic bacteria (1), including a laevan synthetase (8), require high concentrations of salt for their activity, it is possible that the enzymes concerned with cell wall synthesis in *M. halodenitrificans* are halophilic.

The inability of the organism to form cell walls does not explain why calcium and magnesium eliminate stickiness in 0.6 and 0.55 *M* salt and allow growth in 0.3 *M* salt. Lack of stickiness could be the result of DNase activity and vice versa. A calcium-activated DNase has been reported (3), but supernates of non-viscous cultures of *M. halodenitrificans* did not show DNase activity even in the presence of calcium or magnesium. The viscosity of the organism, therefore, does not seem to be related to the inhibition of DNase by lack of calcium. Stickiness of the organism may be affected by calcium or magnesium in other ways. These ions may activate enzymes responsible for cell wall synthesis or they may be essential components of the cell wall. Neither of these explanations seems plausible since the addition of calcium or magnesium does not increase the amount of DAP. In view of the fact that there is considerable swelling and lysis of the cells or "protoplasts" over a narrow range of salt concentration, it seems more likely that the divalent ions are affecting the cytoplasmic membrane or its permeability.

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STUDIES ON THE POLYPHENOL–POLYPHENOLOXIDASE SYSTEM OF WHEAT STEM RUST UREDOSPORES[1]

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Abstract

The presence of a polyphenoloxidase in wheat stem rust uredospores has been demonstrated. The enzyme was released by the germinating spores together with phenolic substrates, resulting in the formation of phenol oxidation products in the surrounding medium. The substrate specificity of the enzyme was investigated, and gallic acid was found to give an unusually high oxidation rate. Pyrogallic acid and catechol were also rapidly oxidized. Toxicity tests have been carried out with phenol oxidation products on germinating spores and it has been shown that toxicity depends on the stage of oxidation. Short-term oxidations led to the formation of highly toxic compounds; longer periods of oxidation converted these into harmless products, whereas very long periods of oxidation resulted again in the production of toxic substances. The spores also contained a quinone reductase which apparently holds the phenolics in a reduced state until they are released during germination or during the infection process. The possible role of the phenol – phenoloxidase – quinone reductase system in the host–parasite relations of the wheat – stem rust complex is discussed.

Introduction

As discussed in detail in a recent paper (5), the defense mechanisms of the host plants against pathogens are often based on an increased activity of polyphenoloxidases and, as a result of this, on this increased accumulation of fungitoxic polyphenol oxidation products in the infected tissues. It was suggested by Newton and co-workers (12, 13) and Kargopolova (7) in the early thirties that the resistance of wheat to stem rust may also be based on the presence, in the resistant host tissues, of higher amounts of phenolic compounds. Actually, the infection of wheat with stem rust is often associated with brown or black discoloration of the infected tissue areas suggesting an accumulation of colored polyphenol oxidation products. However, studies on the enzymology of the wheat plant failed to reveal the presence of polyphenoloxidases in the tissues (9, 20), and the phenol content of wheat leaves, tested by modern methods, is also claimed to be lower than previously suggested (6). These observations seemed to be in contradiction to the original ideas put forward by the Canadian and Russian authors. There remains, however, the possibility that phenoloxidases and phenolic compounds are supplied (secreted) by the fungus component of the host–parasite complex, thus making a polyphenol–polyphenoloxidase reaction possible in the host tissues. The presence in the rust spores of various phenolic compounds that, in addition to the suspected phenolics in wheat, may serve as potential substrates of the enzymatic reaction has recently been demonstrated in this

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laboratory (19). The aim of the present work was to test the spores for the presence and possible excretion of polyphenoloxidases and also to investigate their characteristics as a first and necessary step in the re-examination of the problem of the role of polyphenol–polyphenoloxidase systems in different wheat – rust race combinations. This work was prompted also by recent observations on increased polyphenoloxidase activities in rusted wheat leaf tissues (8, 15).

Materials and Methods

Cell-free Enzyme Preparation

Two grams of uredospores of wheat stem rust (race 15B or 56) were ground in 20 ml phosphate buffer, pH 7.0 (0.067 *M*), in a planetary ball mill for 3 hours at 0° C. Clear, yellowish, cell-free extracts were obtained by centrifuging the ground material in the cold at $5000 \times g$ for 20 minutes. The supernatant was recentrifuged in order to eliminate the traces of oil. Crude extracts were used for the experiments except where indicated in the text. In some cases the characteristics of dialyzed extracts were also examined. Dialysis was carried out at 0° C against 0.067 *M* phosphate or 0.05 *M* versene buffer for 24 hours, with two to three changes of buffer. Extracts were never stored in the refrigerator longer than 2 days before being assayed.

Methods for Enzyme Detection

Enzyme activities were determined spectrophotometrically either in a Coleman or in a Beckman DU spectrophotometer by following the color development due to the oxidation products. Manometric methods were used to check the O_2 uptake.

In the paper chromatographic experiments, the methods described by Van Sumere *et al.* (19) were followed; in some cases distilled water and 2% and 10% acetic acid were also used as developing solvents.

Germination Tests

Germination tests were carried out as previously described (19). Fresh spores collected from infected wheat plants grown in a temperature-controlled greenhouse were used throughout the experiments. Spores were never stored in the refrigerator longer than 24 hours before they were used for the experiments.

Results

Presence of Polyphenoloxidase in Rust Uredospores

As shown in Fig. 1, crude extracts of uredospores rapidly oxidized pyrogallic acid. Somewhat lower, but still remarkable, activities were observed using catechol as substrate. The oxidation of catechol was also demonstrated by coupling the reaction to the reduction of cytochrome *c* (Fig. 2). The reduction of cytochrome *c*, which was followed spectrophotometrically at 550 mμ, was shown to be non-enzymatic.

The polyphenoloxidase activity of extracts was not dependent on the age of the spores (spores collected in 1956, 1957 and fresh spores yielded extracts exhibiting approximately the same activities), and also was not affected by germination. The enzyme in vivo or in the crude state is therefore very stable. This is also shown by the fact that extracts do not lose appreciable activity during a week's storage in the refrigerator or during 2 or 3 days at room temperature. It is of interest that the crude enzyme is protected against inhibition by catechol oxidation products, which inhibition is very characteristic for purified mushroom or potato polyphenoloxidase preparations. This is clearly shown by the low decrease of reaction rate as the reaction proceeds (Fig. 2).

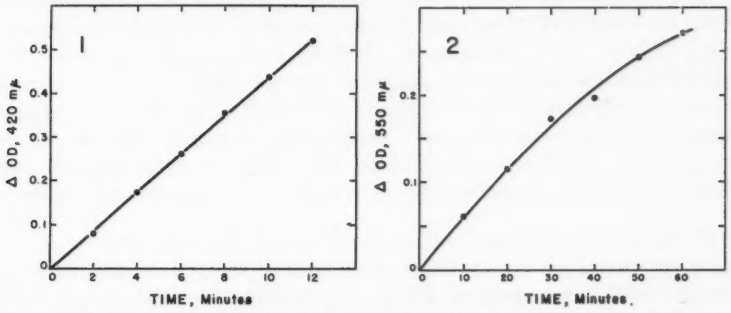

FIG. 1. Oxidation of pyrogallic acid by the polyphenoloxidase of rust spores. Complete system: 1 ml phosphate buffer (pH 7.0) + 1 ml enzyme extract + 1 ml 0.3% pyrogallic acid. Reading taken with a Beckman DU spectrophotometer at 420 mμ.

FIG. 2. Coupling of the oxidation of catechol by the polyphenoloxidase of rust spores to the reduction of cytochrome *c*. Complete system: 1 ml phosphate buffer (pH 7.0) + 1 ml enzyme extract + 0.5 ml 1% catechol + 0.5 ml 3.4×10^{-4} *M* cytochrome *c*. Reaction followed in a Beckman DU spectrophotometer at 550 mμ.

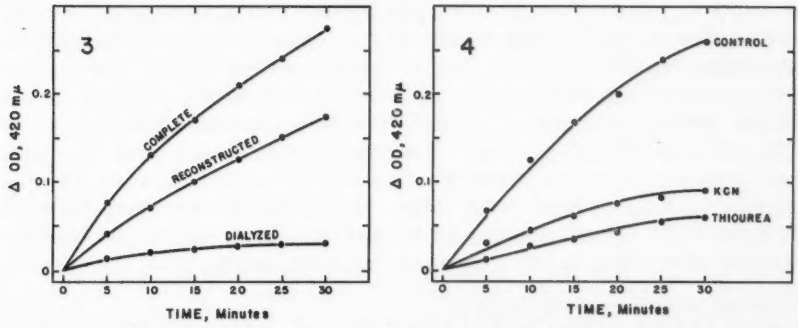

FIG. 3. Effect of dialysis on the polyphenoloxidase activity of stem rust uredospore extracts. Complete system contains 1 ml phosphate buffer (pH 7.0) + 1 ml enzyme solution + 0.5 ml 1% catechol + 0.5 ml distilled water. Reconstructed system contains 1 ml dialyzed extract + 1 ml phosphate buffer + 0.5 ml boiled crude extract + 0.5 ml 1% catechol.

FIG. 4. Inhibition of polyphenoloxidase from rust uredospores by KCN (10^{-3} *M*) and thiourea (5×10^{-4} *M*). Complete system like in Fig. 3.

The enzyme activity was, however, almost entirely lost upon dialysis. The dialyzable factor is heat stable, since activities could be partially restored by adding boiled extracts to dialyzed supernatants (Fig. 3). The enzyme was shown to be sensitive to cyanide and thiourea (Fig. 4).

The enzyme is soluble as sedimentation of the particulate fractions by differential centrifugation (up to 30,000 *g* for 30 minutes) has not resulted in any appreciable decrease in activity.

Tests for the Release of Polyphenolase by Rust Spores

Twenty-five milligrams of uredospores were evenly distributed on the surface of 25 ml distilled water in Petri dishes by means of an electric vibrator. The water was sampled every 12 hours for 3 days and assayed for polyphenoloxidase activity using catechol as substrate. The composition of the reaction mixtures was the same as of those with enzyme extracts. When compared with boiled controls, consistent browning was obtained after 18 to 24 hours of incubation. The reaction was, of course, weaker than that given by enzyme extracts. Microscopic examination revealed that many germ tubes undergo autolysis during this period. The autolysis observed under these conditions may contribute to the release of enzymes by the spores.

Tests for the Release of Phenolic Compounds by Germinating Spores

Since the water on which the spores floated became colored (especially by the end of the experimental period), the possibility existed that substrates for the enzyme were also released and an enzymatic oxidation took place outside the cells. In order to test this hypothesis the solution was filtered, freeze-dried, then taken up in a few drops of ethyl alcohol, and subjected to paper chromatography.

Samples taken from different experiments did not give consistent results. In some chromatograms, for example, spots corresponding to gallic acid could be detected. Most often, however, the substances did not move from the starting line in the various solvent systems tested, including chloroform–formamide, *sec*-butanol–water, 2% and 10% acetic acid and water. The spots resulting from the concentrated filtrate at the starting line were always slightly colored; they gave a strong bluish-white fluorescence in ultraviolet light and in addition they gave the color reactions of phenols with diazotized *p*-nitroaniline, diazotized benzidine, $AgNO_3$ reagent, 1% aqueous $KMnO_4$, 1% $FeCl_3$ + 1% potassium ferricyanide. It appears, therefore, that phenolics are released by the germinating spores and simultaneously are oxidized by the secreted enzymes to polymers of low solubility and mobility.

Substrate Specificity of the Enzyme

A rough qualitative test has been carried out simply by adding suspected substrates to buffered enzyme solutions and observing their discoloration. A method developed by Siegelman (18), consisting of spraying substrate spots on a filter paper sheet with an enzyme solution and incubating the paper for 12 hours in a humid atmosphere, was also used with success. Results,

as summarized in Table I, indicate that catechol, pyrogallol, hydroquinone, and gallic acid are the most rapidly oxidized substrates, as far as it could be estimated from the color development under the circumstances described. Of the most widespread substrates for other polyphenoloxidases, resorcinol and tyrosine were not oxidized.

The substrates that resulted in the most intense discoloration were tested by the manometric method. Gallic acid and pyrogallol were shown to give the highest oxidation rates (Table I). Catechol, despite the appreciable discoloration, gave surprisingly low values of O_2 uptake.

TABLE I

Substrate specificity of the polyphenolase extracted from stem rust uredospores

Substrate	Final substrate concentration, mg/ml	Relative intensity of discoloration (visual)	O_2 uptake in μl/hour by a system containing 0.5 ml phosphate buffer + 1.0 ml enzyme extract + 0.5 ml substrate
Catechol	1.5	++++	8
Hydroquinone	1.5	++	
Pyrogallol	1.5	++++	41
Resorcinol	1.5	−	
DOPA	0.5	+	
Tyrosine	0.5	−	
Chlorogenic acid	1.0	+	10
Gallic acid	1.5	+++	46
Caffeic acid	1.0	−	

Effect of Polyphenol Oxidation Products on Spore Germination

Since both phenolics and polyphenoloxidases are released by germinating spores (which may also take place during the infection process), tests were carried out on the actual inhibitory effect of polyphenols in order to throw some light on the possible role of phenolics in the rust–wheat complex. The effect of various phenolic compounds on rust spore germination has been studied by Anderson (1); however, the compounds used by him were mostly not naturally occurring substances and he did not investigate the most important factor, the role of oxidative processes. To test and compare the effect of phenolics and their oxidation products on spore germination, various substrates were oxidized in test tubes by the polyphenoloxidase extracted from spores (1 ml phosphate buffer + 1 ml substrate in various concentrations + 1 ml enzyme extract). After varying incubation periods the reaction mixtures containing the oxidation products were tested for their effect on germination. Controls were run on phosphate buffer and on the phenolic compounds without oxidation. Experiments with purified mushroom polyphenoloxidase, instead of the crude rust enzyme, gave the same results. When using the mushroom enzyme the complete system contained 10 catechol-ase units/ml. Results are summarized in Table II. It may be seen that catechol was the most toxic of the compounds tested. Short-term oxidations lead to oxidation products that are much more toxic than the original compounds. This is clearly shown if one begins with phenol concentrations

below the toxic concentration level. In this case also we end up in 3 hours with highly toxic oxidation products. Further oxidation converts these toxic compounds into harmless products (16 hours), whereas longer oxidation periods result again in the formation of toxic compounds which, according to chromatographic behavior and color reactions, appear to be polymerized products (melanins).

TABLE II

The effect of polyphenols and polyphenol oxidation products on spore germination

Compound	Concentration, μg/ml	Germination in 90 minutes, %				
		Time of enzymatic oxidation, hours				Average water control, %
		0	3	18	48	
Catechol	500	0	—	—	—	95
	200	3	—	—	—	95
	50	92	8	94	4	95
Hydroquinone	500	0	—	—	—	95
	200	85	—	—	—	95
	50	90	—	—	—	95
Pyrogallic acid	500	0	—	—	—	95
	200	80	—	—	—	95
	50	91	14	97	6	95
Chlorogenic acid	200	93	—	—	—	95
Gallic acid	600	95	6	80	3	95
DOPA	800	92	—	—	—	95

Presence of a Quinone Reductase in Rust Spores

Present-day theories suggest that phenols are held in the reduced state in living tissues by reducing systems inhibiting the accumulation of toxic polyphenol oxidation products. Since rust spores contain both different phenolic substrates and polyphenoloxidase, the presence of some reducing system preventing phenol oxidation was indicated. As a rule, in similar cases, simple reducing substances, e.g. ascorbic acid, were thought to be the major components of the reducing system. More recently the occurrence of quinone reductase enzyme systems have been described (21, 22).

Attempts to couple the oxidation of phenolics to the reduction of TPN as described by Cantino and Horenstein (2) led to the demonstration of a quinone reductase system in rust spores. In contrast to the experiments described by Cantino and Horenstein (2), the extracts from rust spores did not reduce TPN. TPN did not accelerate oxidations, nor did it compete with the oxygen of the air as electron acceptor. On the contrary, the enzymatic discoloration (quinone formation) of the solution was strongly inhibited by TPN, suggesting a decrease in oxidation rates. DPN gave a similar, but lesser effect (Fig. 5).

Since previous studies (17) demonstrated the presence of various enzyme systems able to reduce TPN and DPN in rust spores, the idea emerged that the added coenzymes might act as H carriers reducing quinones in a substrate ⟶ pyridine nucleotide ⟶ quinone reductase ⟶ quinone system.

Therefore, the inhibition of discoloration may be due to a pyridine nucleotide coupled quinone reductase system. To test this hypothesis, reduced triphosphopyridine nucleotide (TPNH) was added to the reaction mixture. The addition of TPNH resulted in a marked decrease in the rate of discoloration, the effect being stronger than that of the oxidized form (TPN) (Fig. 5).

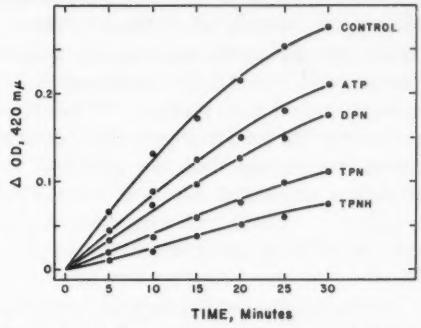

FIG. 5. The effect of pyridine nucleotides on the oxidation of catechol by the polyphenoloxidase of rust spores. The basic composition of the system is identical with that in Fig. 3. Final concentration of the nucleotides 5×10^{-3} *M*.

In order to show that this inhibition is really caused by a quinone reductase enzyme system in the rust spore extracts, the following experiment was conducted. Quinones were prepared enzymatically (pregenerated) by the enzyme plus phenol plus buffer mixture and TPNH was added in higher concentrations to the highly colored solution. As indicated by a marked decrease in color intensity (as compared with boiled controls), the enzyme was able to reduce the pregenerated quinones using TPNH as a H donor. The reducing activity, when the TPNH concentration was kept high enough (10^{-2} *M*), was stronger than the simultaneous oxidation by polyphenoloxidase (Fig. 6).

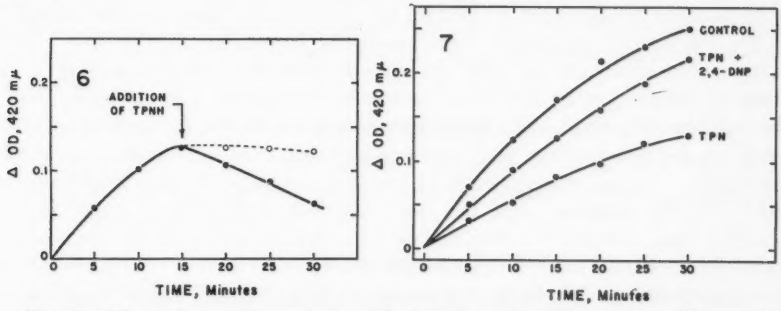

FIG. 6. The presence of a quinone reductase in rust spore extracts. Changes in optical density in a system oxidizing catechol (composition like in Fig. 3) upon addition of 10^{-2} *M* TPNH. Dotted line indicates the course of Δ O.D. in a parallel sample boiled before the addition of TPNH.

FIG. 7. The effect of 2,4-DNP (10^{-4} *M*) on the inhibition by TPN of catechol oxidation by rust polyphenoloxidase. Composition of the system is the same as in Fig. 5.

If the inhibition of enzymatic discoloration by TPN or TPNH is really based on the operation of a quinone reductase, the inhibition should *not* result in a concomitant decrease in O^2 uptake by the system, since the oxidation is continuously going on. Measurements with conventional manometric techniques, in fact, indicated that the TPN inhibition of color development is not correlated with an inhibition of O_2 uptake. This may be regarded as a further indication that the inhibition is due to the quinone reductase activity.

Quinone reductase is an enzyme known to be highly sensitive to uncoupling agents (22). Therefore the effect of 2,4-dinitrophenol (DNP) has also been tested on the rust–polyphenoloxidase system. The crude rust enzyme incubated with catechol + 10^{-4} *M* 2,4-DNP gave stronger discolorations than without uncoupling agent, suggesting that the action of the reductase was inhibited. 2,4-Dinitrophenol interfered also with the inhibitory effect of TPN on the reaction (Fig. 7). These two experiments provided additional evidence for the operation of a quinone reductase in the crude extracted system.

Previous work (3, 10, 11) suggests that ATP inhibits the characteristic browning of injured plant tissues and it has been suggested that this inhibition is correlated with the quinone reductase system (10). A consistent inhibition by ATP of color development was also observed in the case of the rust–polyphenoloxidase system (Fig. 5), which is in agreement with the results presented above suggesting the presence of a quinone reductase in rust spore extracts.

The quinone reductase of rust spores is a soluble enzyme as centrifugation at 23,000×*g* did not sediment the activity. It seems to be less stable than the polyphenoloxidase as its presence could not be demonstrated in 2-year-old spores.

Discussion

The polyphenol–polyphenoloxidase system of rust spores was investigated in view of the possible role of the enzyme system in the host–parasite relations of the wheat–rust complex. Since tissues of cereals usually do not exhibit polyphenoloxidase activity and the phenol content of the wheat leaves was recently reported to be low (6), it seems to be logical to hypothesize that if the polyphenol–polyphenoloxidase system plays any role in the host–parasite relations, its components (enzyme + substrate) must be supplied mostly by the fungus. In fact, the presence in the fungus spores of a polyphenoloxidase has been demonstrated, and the release of the enzyme during germination has also been observed. The release of polyphenoloxidase by germinating spores is associated with a concomitant release of phenolic compounds that are immediately oxidized in the surrounding medium. These observations are in agreement with recent electrophoretic studies by Shaw and Kaul (16) suggesting the presence of oxidized phenolics in extracts from rusted wheat leaves, and also with recent observations on polyphenoloxidase activity in rusted wheat (8, 15).

It has been shown that during the oxidation of various phenolics by polyphenoloxidase preparations, the initial products are highly toxic substances (according to all probability quinones). Further oxidation converts these into harmless products, whereas longer periods of oxidation lead to the accumulation of highly fungitoxic melanins. It is indicated, therefore, that previous views on the *general* fungitoxic character of polyphenol oxidation products cannot be maintained. The toxicity depends on the *stage of oxidation*, this latter being dependent on various factors like the activity of polyphenoloxidases, substrate concentration, activity (concentration) of reducing systems, on the redox potential in the tissues, etc.

Of these factors mentioned above, the reducing systems have been subjected to further studies. Evidence has been presented for the presence of a quinone reductase in rust spore extracts. It seems likely that this enzyme holds the phenolics in reduced state in vivo.

In the light of the above findings it is probable that during the disease development, polyphenols and polyphenoloxidases are released by the fungal hyphae. Resistance and susceptibility, among others, may depend on how far the oxidation of polyphenols released is brought under control in the "new environment" where oxidations are usually going on at a very high rate (4). The amount of simple reducing substances like ascorbic acid, the amount of pyridine nucleotides, the activity of systems reducing TPN and DPN, the activity of quinone reductase using TPNH or DPNH as cofactors, the possible presence of uncoupling substances among the metabolites may all be responsible for the upset of the normal balance. Uncouplers are known, for example, to interfere strongly with the activity of quinone reductases thus favoring the accumulation of oxidized phenolics. Since some of the quinones that arise during the oxidation process are known as uncouplers, the quinone accumulation, once started, may rapidly reach high values (due to a process which is in some sense autocatalytic in nature) resulting in the death of tissue areas and killing the fungus simultaneously (hypersensitivity).

Since all these factors and systems have been found to be present either in the rust spores or in the wheat, a mechanism as outlined above may be very important. Studies along these lines have already been started in several laboratories (8, 14).

Acknowledgments

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AN INTERPRETATION OF THE EFFECTS OF SALTS ON THE LACTIC DEHYDROGENASE OF HALOBACTERIUM SALINARIUM[1]

R. M. BAXTER

Abstract

The lactic dehydrogenase of *Halobacterium salinarium* is unstable and inactive at low solute concentrations. The irreversible loss of activity at low solute concentrations is a first-order reaction, the rate of which decreases with the second or third power of the salt concentration. The enzyme is protected to varying degrees by a variety of inorganic salts and by glycerol or glucose. Potassium chloride and glycerol also protect the enzyme against inactivation by urea.

The enzyme is most active in the presence of potassium chloride, but several other salts are effective to varying degrees as activators. The activation is of the third or fourth order with respect to sodium or potassium chloride concentration. Potassium chloride decreases the affinity of the enzyme for its substrate. Glycerol does not activate the enzyme, but increases its activity in the presence of potassium chloride.

It is suggested that this enzyme differs from enzymes of non-halophilic organisms in being less firmly held in its native catalytically active conformation. At low salt concentrations the electrostatic repulsion between ionized groups on the enzyme expands it to a form which is not catalytically active and which can readily expand further to an irreversibly denatured form.

Introduction

Work in this laboratory (1, 2, 3) and elsewhere (7, 16) has established that many of the enzymes of halophilic bacteria differ from corresponding enzymes of non-halophiles in requiring for maximum activity concentrations of salts which are markedly inhibitory to normal enzymes. In certain moderately halophilic organisms the optimum salt concentrations for individual enzymes differ widely, but in the extreme halophile, *Halobacterium salinarium*,[2] it is probable that all the enzymes require high concentrations of salts (1, 2, 3). It seems likely, therefore, that this peculiarity of the enzymes is part of the physiological basis of bacterial halophilism. In this respect these organisms are analogous to certain thermophiles, of which many of the enzymes (4, 5) and other proteins (13, 14, 20) have been shown to be unusually resistant to heat.

This paper presents an attempt to ascertain the physical basis of the salt requirement of a fairly typical halophilic enzyme, the lactic dehydrogenase of *H. salinarium* (2).

Materials and Methods

The enzyme preparations used were crude sonic extracts prepared as described previously (1, 2, 3). The enzyme was assayed by the manometric ferricyanide technique of Quastel and Wheatley (18). Over the range of

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[2]This organism was referred to as *Pseudomonas salinaria* in previous communications from this laboratory. The name *Halobacterium salinarium* will be used henceforth in accordance with *Bergey's Manual* (7th edition, 1957).

enzyme concentrations used, the activity of the enzyme remained directly proportional to the enzyme concentration. The inactivation of the enzyme at low salt concentrations was studied by mixing a portion of bacterial extract with a suitable volume of water or salt solution in a constant-temperature bath and transferring samples of the mixture, at intervals, to tubes containing potassium chloride solution to prevent further inactivation. Usually the initial volume of the reaction mixture was 30 ml and 3.0-ml samples were taken. The sampling tubes were prepared by adding 3.0 ml of 4 *M* potassium chloride to each, heating to dryness, and adding a further 3.0 ml after cooling. Thus the final concentration after adding the samples and shaking gently to dissolve all the potassium chloride was 4 *M* or more, depending on the salt concentration in the reaction mixture. At salt concentrations as high as this the enzyme is stable almost indefinitely. The lactic dehydrogenase activity of the samples was then measured.

Inactivation by urea was studied by placing weighed amounts of urea in the side arms of the Warburg flasks containing enzyme and other components, and tipping in the urea after gassing and equilibration.

The temperature in all experiments was 29.5° C.

Results

The lactic dehydrogenase of *H. salinarium* is rapidly inactivated at low salt concentrations, and the inactivation probably follows first-order kinetics, since a plot of the logarithm of the residual activity as a function of time yielded a fairly good straight line (Fig. 1). Although the data obtained were sometimes more erratic than those shown here, there were never any systematic deviations indicating a higher order.

The ability of various substances to influence the rate of inactivation of the enzyme is shown in Table I. Since the extract contained sodium chloride it was impossible to determine the rate of inactivation in the total absence of

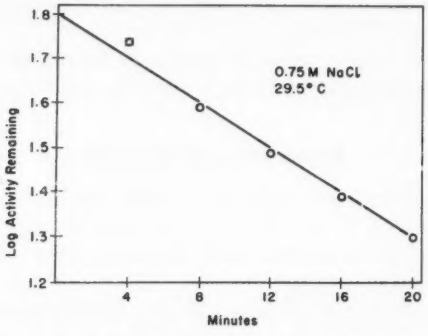

FIG. 1. Kinetics of inactivation of the lactic dehydrogenase of *H. salinarium*. Temperature, 29.5° C. Inactivation allowed to proceed in 0.75 *M* NaCl as described under "Methods". Residual activities assayed in presence of 0.016 *M* sodium lactate. Average of two experiments.

salts or in the presence of other salts alone. The lowest sodium chloride concentration that could be tested was 0.5 *M*. Increasing the total salt concentration to 1.0 *M* by the addition of sodium chloride, potassium chloride, or lithium chloride decreased the rate of inactivation four or five times. Ammonium chloride was perhaps a little less effective, and calcium chloride considerably more effective at the same ionic strength. The ability to decrease the rate of inactivation of the enzyme is, therefore, not a specific property of any particular salt. The rate of inactivation was decreased by glycerol, and still more by glucose, but was increased by ethanol.

TABLE I

Protection by solutes of the lactic dehydrogenase of *H. salinarium*

Solute and molar concentration	Half life of enzyme, minutes
NaCl, 0.5	6
NaCl, 1.0	26
NaCl, 0.5 + KCl, 0.5	30
NaCl, 0.5 + LiCl, 0.5	20
NaCl, 0.5 + NH_4Cl, 0.5	15
NaCl, 0.5 + $CaCl_2$, 0.17*	About 60
NaCl, 0.5 + glycerol, 1.0	10
NaCl, 0.5 + glucose, 1.0	16
NaCl, 0.5 + ethanol, 1.0	2

*This concentration was chosen because it gives the same ionic strength as 0.5 *M* NaCl or other monovalent salt.

It has been suggested (19) that solutes promote the growth of certain microorganisms by lowering the activity of water in the medium. Since water is known to be a fairly effective protein denaturant (12), it seemed possible that solutes may inhibit the inactivation of the enzyme by acting simply as inert diluents of water rather than by exerting a protective effect on the enzyme. To test this possibility, the effect of potassium chloride and glycerol on the inactivation of the enzyme by urea, a known powerful denaturant, was determined. When urea was tipped into the main compartment of the Warburg flask, at the beginning of an experiment, the rate of gas evolution did not remain constant throughout the experiment but declined as the enzyme was inactivated. The rates of decline of activity under different conditions were taken as a qualitative indication of the rates of inactivation. Since potassium chloride and glycerol influenced the activity of the enzyme as well as its stability, the results have been presented by plotting the gas outputs in the presence of urea as percentages of the total gas outputs of corresponding controls with the same concentration of potassium chloride or glycerol (Fig. 2). The protective effect of potassium chloride and glycerol is shown by the less rapid decline in the slope of the curve in the presence of 2.38 *M* KCl or 1.5 *M* KCl and 1.4 *M* glycerol than in the presence of 1.5 *M* KCl alone. It seems clear, therefore, that potassium chloride and glycerol exert a genuine protective effect on the enzyme.

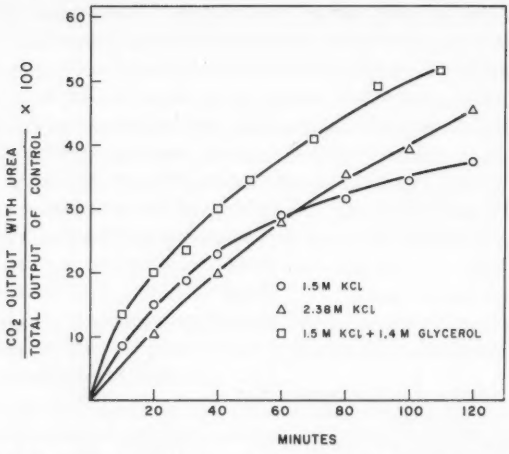

FIG. 2. Effect of potassium chloride and glycerol on the inactivation by urea of the lactic dehydrogenase of *H. salinarium*. Crystalline urea added from side arm to give a concentration of 4.0 *M*. Sodium lactate, 0.016 *M*; temperature, 29.5° C.

Inhibition of the inactivation of enzymes by substances capable of reacting reversibly with them (activators, substrates, or certain inhibitors) is often observed, although differences in stability between the protected and unprotected forms of the magnitude described here have not, to my knowledge, been reported previously. To describe effects of this kind Van Eys *et al.* (21) have derived an equation which may be written as follows: $\log (k/k_p - 1) = \log K + n \log [P]$ where k is the velocity constant for the inactivation of the enzyme in the absence of the protecting substance, k_p is the constant in the presence of protector at any concentration P, n is the number of moles of protector reacting with 1 mole of enzyme to yield the protected form, and K is the reciprocal of the equilibrium constant for this reaction. This relationship cannot be applied directly to the lactic dehydrogenase of *H. salinarium* because k cannot be determined, but since at most salt concentrations $k \gg k_p$, $k/k_p \gg 1$ and it is approximately true that $-\log k_p = \log K - \log k + n \log [P]$. Thus by plotting $\log k_p$ against $\log [P]$ a straight line should be obtained of slope n. Figure 3A shows data treated in this way, and it can be seen that n, the number of moles of salt required to protect 1 mole of enzyme, is between two and three.

Since the protective effect of salts did not appear to be specific, it seemed likely that an explanation was to be found in terms of general ionic effects on the enzyme protein. Since proteins are composed of flexible molecules carrying large numbers of charged groups, anything affecting the mutual interactions of these charges may profoundly influence the size and shape of the protein molecules (15). In particular, the excess positive or negative charges which the molecules will carry except at the isoelectric point will tend mutually to repel one another. Since denaturation is generally believed

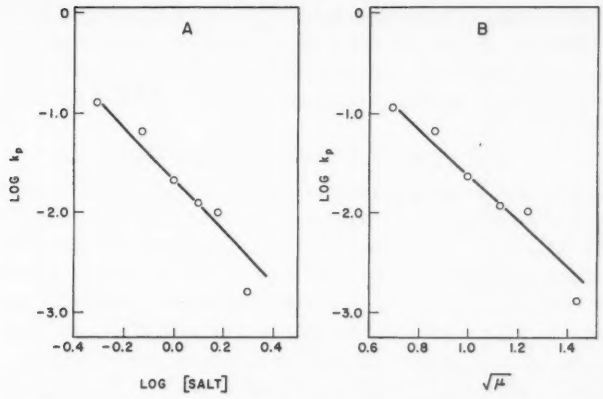

FIG. 3. Influence of sodium chloride on the rate of inactivation of the lactic dehydrogenase of *H. salinarium*. Logarithms of the first-order velocity constants of the inactivation as a function of the logarithms of the salt concentration [(A)], and as a function of the square roots of the ionic strength [(B)].

to involve an unfolding of polypeptide chains, the greater the internal electrostatic repulsions the greater will be the tendency of the protein to suffer denaturation. According to the theory of Debye and Hückel the presence of salts will tend to reduce the electrostatic effects and thus decrease the likelihood of denaturation. It can be shown (17) that for reactions between ions in solution the velocity constant k is related to the ionic strength μ according to the following relationship:

$$\log k = qZ_aZ_b\sqrt{\mu} - \log k_0$$

where k_0 is the velocity constant in the absence of salts, Z_a and Z_b are the charges on the reactants, and q is a constant which under the conditions used here is roughly unity. The derivation of this equation involves certain approximations valid only at low ionic strengths, but it should predict the general magnitude of effects observed at higher concentrations. Yon (23), studying effects somewhat similar to those discussed here, found this relationship to be obeyed up to an ionic strength of 1.7. Since we are concerned with the separation rather than the combination of electrostatic charges, the term involving ionic strength will be negative.

Data plotted according to this equation yielded a fairly good straight line, the slope of which is about three (Fig. 3B). Since the slope is roughly equal to the product of the charges of the interacting groups, this low value indicates that loss of catalytic activity is associated with disruption of only a small portion of the enzyme molecule.

If the enzyme required salt only to prevent inactivation, then the initial rates of lactate oxidation should be independent of salt concentration and the only effect of salt would be to decrease the rate of decline of the activity of the enzyme. However, this does not occur; the activity as well as the stability

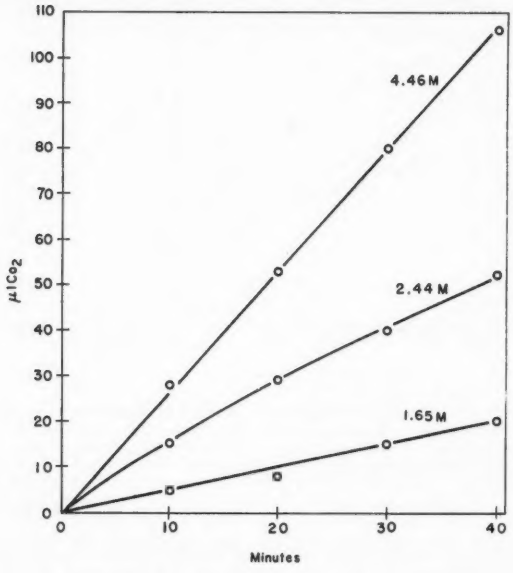

FIG. 4. Action of the lactic dehydrogenase of *H. salinarium* at three sodium chloride concentrations. Sodium lactate concentration, 0.016 *M*; temperature, 29.5° C.

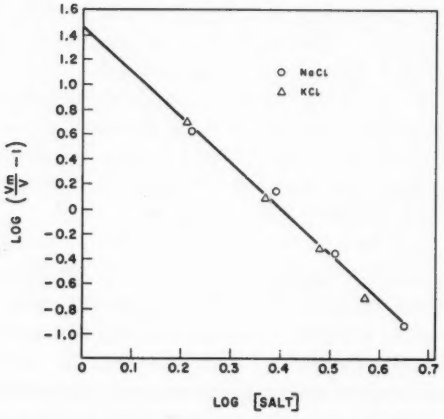

FIG. 5. Effect of sodium chloride and potassium chloride on the activity of the lactic dehydrogenase of *H. salinarium*. Sodium lactate concentration, 0.016 *M*; temperature, 29.5° C.

of the enzyme is lower at lower salt concentrations (Fig. 4). The number of moles of salt required to activate 1 mole of the enzyme can be determined in a manner analogous to that used in determining the molar ratio involved in protection of the enzyme. For an enzyme requiring an activator it can be shown that

$$\log(Vm/V-1) = \log K - m \log [C]$$

where Vm is the velocity of the reaction when all the enzyme is in the active form, V is the velocity at any concentration C of activator, m is the number of moles of activator required to convert 1 mole of enzyme to the active form, and K is the reciprocal of the equilibrium constant for the reaction between enzyme and activator, and is analogous to a Michaelis constant. When the function $\log(Vm/V-1)$ was plotted against the logarithm of the salt concentration, a reasonably good straight line was obtained, with a slope between three and four (Fig. 5). Thus activation of the enzyme seems to require 3 to 4 moles of salt per mole of enzyme, about one more than is required for protection.

The activating effect of salts is only slightly more specific than the protective effect. A number of salts are capable of activating the enzyme, and the rate of the reaction is influenced by the nature of both the cation and the anion (Table II). Since high concentration of salts of weak acids or bases interfered with the buffer system it was difficult to obtain meaningful data on the effects of these salts on the activity of the enzyme. It is probable that ammonium salts are incapable of activating the enzyme; the enzyme was not active in the presence of ammonium chloride, and in the presence of potassium chloride the same concentration of ammonium chloride inhibited the enzyme, but not completely. Glycine behaved in the same way as ammonium chloride. It seems likely, therefore, that the absence of activity in the presence of these substances was not merely the result of interference with the buffer. Salts of divalent cations interfered so badly with the buffer that no information could be obtained.

Glycerol alone did not activate the enzyme, but it increased the activity of the enzyme in the presence of potassium chloride; the presence of 2.6 M glycerol increased the activity of the enzyme in 1.75 M KCl by about 65%.

TABLE II

Activation of the lactic dehydrogenase of *H. salinarium* by salts

Salt	Molar concentration	CO_2 evolved per hour,* μl
KCl	1.61	54
KCl	2.63	130
KNO_3	2.63	41
KBr	2.63	77
NaCl	2.63	96
$NaNO_3$	2.63	12
LiCl	2.63	87
$(C_2H_5)_4NBr$	2.63	2
Na_2SO_4	1.37	37

*Ferricyanide technique (18).

The activating effect of salts is opposed by a decreased affinity of the enzyme for its substrate at higher salt concentrations. Figure 6 shows the effect of substrate concentration on the activity of the enzyme at two concentrations of NaCl. The data have been plotted as suggested by Eadie (6), that is, the velocity of the reaction, V, has been plotted against $V/[S]$, where [S] is the substrate concentration. It can be shown that this yields a straight line if the system follows Michaelis–Menten kinetics. The slope of the line represents the Michaelis–Menten constant, Km, which is inversely proportional to the affinity of the enzyme for its substrate, and the intercept on the V axis represents Vm, the velocity when the enzyme is saturated with substrate. It can be seen that Km increases along with Vm as the salt concentration increases. This cannot be explained on the basis of any simple kinetic model (9) and probably the effect of salts on Km and on Vm involve different mechanisms. All other experiments described here have been performed using sufficiently high substrate concentrations to saturate the enzyme so that changes in Km will not affect the rate of the reaction.

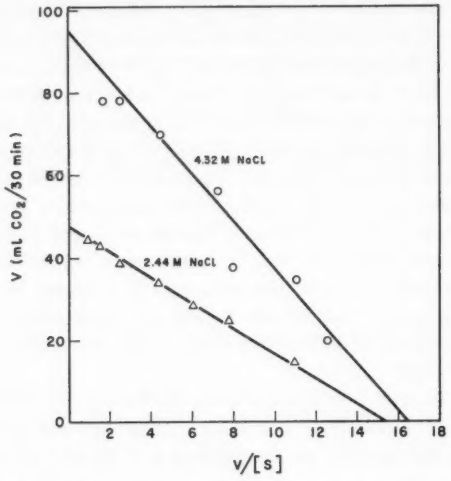

FIG. 6. Effect of sodium chloride concentration on the Michaelis constant of the lactic dehydrogenase of *H. salinarium*.

Discussion

It seems safe to assume that the inactivation which the lactic dehydrogenase of *H. salinarium* undergoes on dilution is a reflection of denaturation of the protein. The observation that the inactivation follows first-order kinetics makes it unlikely that irreversible aggregation of the enzyme is involved for instance. When such properties as viscosity or optical rotation are used as criteria of protein denaturation, orders higher than one are often observed; this is explained by the assumption that the over-all process involves several different reactions proceeding at different rates (12). The loss of enzymatic

activity, however, probably involves only a simple one-step alteration of the protein structure, and this might be expected to follow first-order kinetics. The suggestion that only a slight structural change is involved is supported by the relationship between rate of inactivation and salt concentration (Fig. 3B).

The fact that the inactivation of the enzyme by urea is inhibited by substances that prevent inactivation by dilution further supports the view that the latter represents denaturation of the protein. It also shows that protective substances act by some direct effect on the protein and not by simply diluting the water.

Although the activating effect of salt can be distinguished experimentally from the protective effect, the two are sufficiently similar to suggest that both have the same basis. Activation requires about one mole more of salt per mole of enzyme than does protection. This seems to indicate that interaction of the enzyme with salt leads first to a stable but inactive form of the protein, and only at higher concentrations to the active form. The activation of the enzyme is more specific with respect to salt than is the protection. It is noteworthy that activity is greatest with potassium chloride, that is with the cation and anion of the smallest hydrated radius of those tested. It seems plausible that the lowered activity with other ions represents steric hindrance of the combination of enzyme and substrate when the enzyme is associated with bulky ions. This moderate degree of specificity with respect to both cation and anion distinguishes the activation of this enzyme from more familiar activations, which are generally rather highly specific with respect to either ion, but not both.

The essentially equal effectiveness of the alkali chlorides in protecting the enzyme is to be expected if the effect is a purely electrostatic one. The greater effectiveness of calcium chloride suggests that the calcium ion may form salt linkages with anionic groups, thus neutralizing them completely, and perhaps also form cross linkages between adjacent groups. The observation that the relationship between stability of the enzyme and salt concentration is of the type predicted on the basis of the Debye–Hückel theory cannot be regarded as conclusive because it is uncertain to what extent the theory is applicable at the high salt concentrations involved. It is, however, extremely suggestive when considered along with the other evidence.

The increase in the Michaelis constant with increasing salt concentrations probably indicates that formation of the enzyme–substrate complex involves formation of an ionic bond between the ionized carboxyl group of lactate and a cationic group on the enzyme. The Debye–Hückel theory predicts that such a reaction should be inhibited on increasing the ionic strength. The effect of salts on the hydrolysis by trypsin of heat-denatured lactoglobulin has been explained on this basis (23).

The protective effects of glycerol and glucose cannot be explained in terms of electrostatic effects. These substances protect many proteins against denaturation and no satisfactory explanation has to our knowledge been

suggested. There seems to be no reason to assume that they should act in the same way as salts. No explanation can be suggested for the ability of glycerol to increase the activity of the enzyme in the presence of potassium chloride.

The data presented in this paper suggest that halophilic enzymes differ from other enzymes in being rather loosely held in their native, enzymatically active conformations, so that it is only when the intramolecular electrostatic repulsions are reduced by the presence of salts that they are able to assume the structure in which they are active as catalysts. On lowering the salt concentration they first expand reversibly to an inactive conformation, and with further lowering of the salt concentration irreversible disruption of the molecule may occur.

It is likely that the difference between halophilic and non-halophilic enzymes is quantitative rather than qualitative since there are many reports in the literature of effects of salts on non-halophilic enzymes similar to those reported here, but involving salt concentrations about an order of magnitude lower (8, 11, 22). The cholinesterase of the housefly is particularly interesting since this enzyme showed increased stability, increased activity, and decreased affinity for its substrate in the presence of salts (22). It seems likely that alterations in stability of enzymes and other proteins, arising as a result of alterations in their fine structure, are not uncommon in nature and may play an important part in enabling certain organisms to exist in unusual environments. Thus the flagellar protein of certain thermophilic bacteria is unusually firmly held by hydrogen bonding in its native configuration (14). Structural alterations of this type probably occur fairly readily; many thermophilic organisms can produce enzymes of differing heat stability depending on the temperature at which they are grown (4, 5), and the mutation of a single gene in *Neurospora* can increase manifold the heat stability of its tyrosinase (10). It seems possible that halophiles may have arisen by natural selection of mutants possessing enzymes of unusually loose structure and, consequently, increased ability to function in the presence of salts.

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GLUCOSE METABOLISM IN PENICILLIUM DIGITATUM[1]

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Abstract

The utilization of glucose by proliferating *Penicillium digitatum* cells has been examined by the radiorespirometric method. Glucose is catabolized by this organism mainly (77%) by way of the Embden–Meyerhof–Parnas pathway in conjunction with the tricarboxylic acid cycle and CO_2 fixation processes. A minor portion (23%) of the administered glucose is utilized via an oxidative route involving a C_1–C_5 cleavage. Pathway estimations on the basis of CO_2 production and glucose consumption were also discussed.

Introduction

While the nature of glucose metabolism in *Penicillium chrysogenum* has been studied by several workers (1, 2, 3, 7, 8), the metabolic processes for glucose utilization in the citrus fruit mold, *Penicillium digitatum*, have received very little attention. Recent communications from this laboratory (5, 12) have identified some of the catabolic pathways for the metabolism of carbohydrate breakdown products in *P. digitatum*. Included was the role played by acetate and CO_2 for the biosynthesis of aspartic acid and glutamic acid in this organism.

In the past years, numerous methods employing radioactive techniques were developed to identify and estimate the catabolic pathways of carbohydrate in biological systems, although some of them were subjected to limitations (13). In the present work, the radiorespirometric method of Wang *et al.* (11) has been applied to the study of glucose catabolism in *P. digitatum*. Estimation of individual pathways was presented and discussed in light of the equation presented by Korkes (4). The latter was shown to be closely related to the equation of Wang *et al.* in basic principles.

Experimental Method

Isotopic Substrates

Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were furnished by the Bureau of Standards through the kind co-operation of Dr. H. S. Isbell. Glucose-U-C^{14} was obtained from Tracerlab Inc. Glucose-3,4-C^{14} was prepared in this laboratory according to the method of Wood and co-workers (14) and was shown to be chromatographically pure.

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Incubation Procedures

The organism used in the present study is the same as described previously (5). The initial growth phase was conducted in a 3-liter, three-necked flask equipped with a gassing sparger, a sampling tube, and a gas outlet. The incoming air was passed through a sulphuric acid scrubber and a sterile cotton plug before being sparged into a growth medium of the following composition: glucose, 13.2 g; asparagine monohydrate, 3.0 g; casamino acids (Difco), 4.0 g; ammonium tartrate, 5.0 g; 20 ml of salts mixture (consisting of $NH_4H_2PO_4$, 1.0 g; $NaNO_3$, 1.0 g; $MgSO_4$, 0.25 g; KCl, 0.10 g; $CaCl_2$, 0.10 g; $FeCl_3$, 5.0 mg; $MnCl_2$, 0.10 mg; KI, 0.01 mg; $ZnCl_2$, 0.05 mg; H_3BO_3, 0.05 mg; $CuCl_2$, 0.01 mg); and 1.0 ml of a vitamin solution (containing thiamine hydrochloride, 0.4 mg; calcium pantothenate, 0.6 mg; and pyridoxine hydrochloride, 0.8 mg). The mixture was diluted to 1 liter with distilled water, adjusted to pH 3.5–3.6 and 6 *N* HCl, and autoclaved at 15 p.s.i. for 30 to 40 minutes (15).

The above growth medium was inoculated with spores from fresh spore stock cultures grown on potato-dextrose agar slants. Growth was continued for 40 to 48 hours under adequate aeration, the mycelia were then harvested by centrifugation, washed three times, and finally suspended in a carbon-free medium which was identical with the growth medium except that glucose, asparagine, ammonium tartrate, and the casamino acids were omitted.

Radiorespirometric Experiments

The apparatus used in the radiorespirometric studies is essentially the same as that described by Wang *et al.* (11). A battery of incubation flasks was placed in a constant-temperature bath, each flask containing a given amount of cell suspension, being equipped with a gas sparger, a sampling tube, and a gas outlet which was connected to a CO_2 trap containing 0.5 *N* CO_2-free NaOH solution for the collection of respiratory CO_2. The rate of aeration for each flask was controlled by means of a master flowmeter, connected to each incubation flask through a bypass manifold. Labelled substrate was added after cells were allowed to deplete for 1 hour under aeration. The solution in each CO_2 trap was replaced hourly and the carbonate was recovered as $BaCO_3$ for radioactivity assays.

Radioactivity Measurement

Radioactivity of respiratory CO_2 in the form of $BaCO_3$ was measured with a thin window Geiger counter. Counting data were corrected for background and self-absorption in a conventional manner. C^{14} contents in cells and incubation media were assayed by converting carbonaceous matter in the sample to $BaCO_3$ by means of wet combustion and they were counted in a manner similar to that employed for respiratory CO_2. For the determination of the amount of glucose incorporated into the cellular polysaccharides, labelled cells were hydrolyzed with 0.05 *N* HCl and Dowex-50 resin according to the method of Paulson *et al.* (6). The solubilized polysaccharides were separated from the resin by filtration and their radioactivity determined in a manner similar to that used for the incubation media.

Results and Discussion

Nature of the Endogenous Respiration

Molds are generally considered to have a greater endogenous respiration rate than most other microorganisms. This fact is also demonstrated by findings in the present work. Since the rate of C^{14} isotope appearing in the respiratory CO_2 from a C^{14}-labelled substrate is dependent to a considerable extent upon the amount of the concurrent endogenous respiration prevailing in this organism, high endogenous respiration is reflected by the observed rate of conversion of substrate activity to the respiratory CO_2, as shown in Fig. 1. The slopes of the $C^{14}O_2$ interval activity recovery curves were considerably less steep than observed with *Saccharomyces cerevisiae*, which is known to have a rather low endogenous respiration (10, 11).

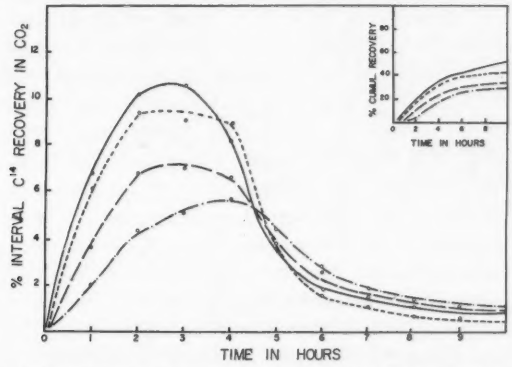

FIG. 1. Interval radiochemical recovery in respiratory CO_2 from 44-hour-old culture of *P. digitatum* grown on specifically C^{14}-labelled glucose.
Substrates: glucose-1-C^{14} ——, glucose-2-C^{14} — — — —, glucose-3,4-C^{14} – – – –, glucose-6-C^{14}—·—·—· Level of substrate per flask, 90 mg; level of radioactivity per flask, 0.5 microcurie; time for medium glucose disappearance, 4 hours; weight of cells per flask (dry weight), 250 mg; air-flow rate per flask, 400 cc per minute; temperature, 30° C.

Experiments utilizing cells grown on glucose-U-C^{14} exclusively from the spore stage indicate that the endogenous respiration of *P. digitatum* is suppressed by exogenous glucose to an extent equivalent to one fourth of the rate in the absence of the substrate (7). This strongly suggests, as do the respiratory $C^{14}O_2$ recovery curves, that a considerable amount of labelled substrate glucose is routed through the endogenous pool and is metabolized to respiratory CO_2 at a somewhat slower rate with considerable dilution of the C^{14} isotope. Further studies are in progress at the present time to determine the amount of inhibition caused by the presence of different substrate carbohydrates.

The Identification of the Catabolic Pathways of Glucose

The relative rates whereby individual carbon atoms of glucose are converted to CO_2 are given in Fig. 1. Data presented represent the average of findings of several identical experiments. The preferential combustion of C-1 to

CO_2 in comparison with C-6 of glucose undoubtedly reflects the operation of a catabolic pathway of glucose other than the glycolytic scheme in this organism. This conclusion is drawn from the fact that the operation of glycolysis in combination with a secondary oxidative process, such as the tricarboxylic acid cycle, would result in equal rates of conversion of C-1 and C-6 to CO_2.

By examining the known information on the catabolic pathways of glucose in microorganisms, one is left with the impression that the basic mechanisms for the breakdown of glucose can be crudely classified into two categories, namely: a C_1–C_5 cleavage to provide pentose and related compounds which are further oxidized by several possible mechanisms, such as pentose cycle, etc.; and a C_3–C_3 cleavage giving rise to C_3 units which are in turn routed through tricarboxylic acid cycle processes in many cases. The latter version of degradation is understood to fashion itself either in the nature of the Embden–Meyerhof–Parnas (EMP) glycolytic pathway or the Enter–Doudoroff pathway, in which, instead of triose, a mole of pyruvate is produced from the upper three carbon atoms of glucose with C-1 corresponding to the carboxyl carbon atoms of the pyruvate. It is also interesting to note that existing literature indicated that these two types of C_3–C_3 cleavage generally do not operate concurrently in microorganisms.

In the present case, our experimental results point to the operation of the EMP glycolytic pathway for the production of C_3 units from glucose. The conclusion is drawn from the fact that the radiochemical recovery of C-3,4 in CO_2 (41%, Table I) is noticeably greater than the average recovery of C-1 and C-6 in CO_2 (38%), while they should be equal to each other when one assumes that the Entner–Doudoroff pathway is the sole pathway in this organism. This is further supported by the observation that C-1 and C-6 of glucose were incorporated into cellular constituents to a much greater extent than that from C-3,4 of glucose as shown in Table I. Earlier, the operation of the EMP glycolytic pathway in a related organism, *Penicillium chrysogenum*, has been reported by Koffler and Heath (3). The foregoing discussion thus led us to believe that an oxidative pathway involving a C_1–C_5 cleavage of glucose is probably operative in *P. digitatum* which is responsible for the observed preferential recovery of C-1 of glucose in the respiratory CO_2.

TABLE I

Fate of carbon atoms of glucose in proliferating *P. digitatum*

Substrates	Percentage recovery			
	Respiratory CO_2	Cells	Fermentation products (med.)	Total
Glucose-1-C^{14}	46	50	3	99
Glucose-2-C^{14}	35	59	4	98
Glucose-3,4-C^{14}	41	36	6	83*
Glucose-6-C^{14}	30	54	14	98

*Poor recovery due to difficulties encountered in the radiochemical assay of incubation medium.

The fate of the C_5 unit (pentose) produced in the C_1–C_5 cleavage process cannot be clearly understood in the present experiment. However, since the radiochemical recovery of glucose-2-C^{14} in CO_2 is considerably lower than that of glucose-1-C^{14}, it is reasonable to believe that the bulk of pentose is routed for biosynthetic functions. Existing information on pentose metabolism includes the direct incorporation of the intact molecule as well as various cleavage processes giving rise to C_2 and C_3 units. The C_2 unit is thought to correspond to C-1 and C-2 of ribose. It is interesting to note that in the present work, C-2 of glucose was found to be incorporated into cellular constituents, particularly amino acids, in significantly greater amount than that of C-6 in repeated experiments. In view of the demonstrated role played by the "active C_2 unit" in biosynthetic functions of this organism (5), it is possible that the cleavage of pentose may serve as one of the major processes in furnishing the "active C_2 unit".

The mechanism by which C-6 of glucose is converted to respiratory CO_2 appears to be due to glycolysis in combination with an active tricarboxylic acid cyclic process (5). Since glucose-2-C^{14} is converted to respiratory CO_2 to a greater extent than glucose-6-C^{14}, it can be also assumed that very little randomization between C-1 and C-6 of glucose by way of reversed aldolase reaction, which would result in the direct conversion of C-6 to CO_2, has occurred.

Quantitative Estimation of Pathways

The quantitative estimation of the relative contributions made by the EMP glycolytic pathway and the C_1–C_5 cleavage pathway in the utilization of glucose by *P. digitatum* has been made according to the method of Wang *et al.* (10). The equations for these calculations made use of the cumulative radiochemical recoveries of metabolic $C^{14}O_2$ from each labelled glucose at the time substrate glucose was completely consumed by cells, 4 hours in the present experiment, and are derived primarily on the basis of the following assumptions: (*a*) glucose is metabolized to CO_2 either by an oxidative C_1–C_5 cleavage of glucose, or the EMP glycolytic pathway in combination with the tricarboxylic acid cycle; (*b*) trioses derived from glucose are equivalent to each other with respect to further metabolic reactions and they are metabolized by way of either oxidative decarboxylation to form C_2 compounds (such as acetate) or incorporated into cellular constitutents without disruption of the C_3 skeleton. The validity of this assumption is indicated by the extent of incorporation of substrate activity into cellular constituents in the glucose-3,4-C^{14} experiment (Table I) and the active operation of a CO_2 fixation process in this organism reported in a previous paper (5); (*c*) preferential conversion of C-1 of glucose to respiratory CO_2 via C_1–C_5 cleavage is a rapid process; (*d*) the randomization of glucose skeleton during active cellular catabolism is limited in extent. In the course of rapid utilization of glucose by a proliferating organism, it is reasonable to assume that the forward reactions of glucose breakdown are greatly favored over the reversed reactions which are responsible for the reformation of glucose with a randomized skeleton. Con-

sequently, unless the organism is permitted to incubate with the specifically labelled glucose for a prolonged period (3), the randomization of glucose skeleton is not likely to be a serious problem in radiorespirometric studies.

If these assumptions are borne in mind, the contribution of the individual pathway for the metabolism of administered glucose can then be made in the following manner; let G_1 and G_6 represent the cumulative radiochemical recovery of substrate activity in respiratory CO_2 at the time of substrate glucose exhaustion within the cells. The fraction of glucose catabolized via the C_1–C_5 cleavage pathway, G_p, is:

$$G_p = (G_1-G_6)/(G_T-G_T') \qquad [1]$$

where G_T is the total activity of each labelled substrate administered to the medium, and G_T' is the amount of glucose substrate activity which has been determined experimentally as incorporated into the polysaccharide and presumably does not involve either pathway. In the case of yeast (10) it was not necessary to employ the correction factor G_T', since it has been shown that the endogenous reserve in yeast is rather low, while in the *P. digitatum*, as has also been shown with *P. chrysogenum* (9), a high endogenous reserve is present which necessitates the foregoing correction.

Since it has been assumed that only two pathways are operative in this organism, the fraction of the glucose utilized by the EMP pathway, G_e, is therefore:

$$G_e = 1-G_p. \qquad [2]$$

Recently, the application of radioactive tracer techniques in estimating pathway participations has been critically examined by Korkes (4). In a set of equations presented by this author, the specific activities of respiratory CO_2 from a biological system metabolizing glucose-1-C^{14} and glucose-6-C^{14} were used for the calculation of the fraction of CO_2 derived from individual pathways. The correlation of the equations of Korkes with equations [1] and [2] in the present report, which represent the fraction of glucose catabolized via different pathways, can be illustrated as follows:

G_1, G_2, $G_{3(4)}$, G_6 = radioactivity recovered in respiratory CO_2 from cells metabolizing equal amounts of glucose-1-, 2-, 3-(or 4-), or 6-C^{14}, respectively, taken at the time of complete exhaustion of administered glucose (c.p.m.).

G_T = total radioactivity of each labelled substrate administered (c.p.m.).

G_T' = radioactivity of substrate activity incorporated into the polysaccharide fraction of the cells (c.p.m.).

A = specific activity of the labelled glucose (c.p.m./mM).

G = fraction of respiratory CO_2 derived via EMP–TCA pathway.

S = fraction of respiratory CO_2 derived via a C_1–C_5 cleavage pathway.

G_e = fraction of administered glucose catabolized via the EMP–TCA pathway.

G_p = fraction of administered glucose catabolized via a C_1–C_5 cleavage pathway.

W = weight of respiratory CO_2 from cells metabolizing glucose at the time when substrate glucose is completely consumed by cells.

R = ratio of specific activities of respiratory CO_2 from cells metabolizing glucose-6-C^{14} over that from cells metabolizing glucose-1-C^{14}.

N = dilution factor for C-1 or C-6 of glucose in CO_2 via the EMP–TCA pathway. When all carbon atoms of glucose are completely converted to CO_2, the specific activity of C-6 in glucose-6-C^{14} is diluted with five unlabelled carbon atoms and hence the value of N reaches its minimum of 6 (4). In the case of growing cells, carbon atoms of glucose are incorporated to a variable extent into the cellular constituents, the dilution factor for C-6 of glucose-6-C^{14} in CO_2 can then be estimated as:

$$N = (2G_2+2G_{3(4)}+2G_6)/G_6$$

under the assumption that C-2, C-3, C-4, and C-6 of glucose are oxidized to CO_2 via the EMP–TCA pathway exclusively.

P = extent to which carbon atoms of glucose are oxidized to CO_2 via the EMP–TCA pathway, which can be expressed as:

$$P = (2G_6+2G_2+2G_{3(4)})/6\{(G_T-G_T')-(G_1-G_6)\}.$$

The numerator represents the recovery of carbon atoms of glucose in CO_2 via the EMP–TCA pathway and the denominator represents the fraction of glucose catabolized via the EMP–TCA pathway.

Pathway participations with respect to the origin of CO_2 (4):

$$R = (G_6/W)/(G_1/W) = (AG/N)/\{(AS/1)+(AG/N)\} \quad [3]$$

and,

$$S = (1-R)/\{1+(N-1)R\} \quad [4]$$

$$G = 1-S = NR/\{1+(N-1)R\}. \quad [5]$$

Pathway participations with respect to glucose utilization:

$$G_p = S/\{S+(G/6P)\}. \quad [6]$$

Substituting value of S (equation [4] and G (equation [5]) into equation [6]:

$$G_p = \frac{(1-R)/\{1+(N-1)R\}}{[(1-R)/\{1+(N-1)R\}] + [NR/\{1+(N-1)R\}]\,6P} \quad [7]$$

$$= (1-R)/\{(1-R)+(NR/6P\}.$$

Substituting values of R, N, and P as defined into equation [7], we obtain:

$$G_p = (G_1-G_6)/(G_T-G_T')$$

which is the same expression as equation [1].

The pathway participations with respect to both the origin of CO_2 and the utilization of glucose are given in Table II. It can be seen from these results and the derivation inherent in each of the equations involved in the calculations that glucose catabolism in a given microbial system is represented better by the estimation of pathway participation based on the utilization of glucose.

Thus, in *P. digitatum*, although 11% of the respiratory CO_2 is derived via a C_1–C_6 cleavage pathway, actually the administered glucose was utilized to an extent of 23% by way of the nonglycolytic route. The main portion is presumably catabolized via the EMP–TCA pathway.

TABLE II

Relative participation of pathways for glucose catabolism in *P. digitatum*

	Symbol	Radioactivity, c.p.m.
Substrate recovery		
Total glucose administered	G_T	8.65×10^5
Recovery of intact glucose in cellular polysaccharides	G_T'	1.73×10^4
Recovery of glucose carbon atoms in respiratory CO_2		
Glucose-1-C^{14}	G_1	3.10×10^4
Glucose-2-C^{14}	G_2	2.07×10^4
Glucose-3,4-C^{14}	$G_{3(4)}$	2.85×10^4
Glucose-6-C^{14}	G_6	1.49×10^4

Pathway	Symbol	Participation percentage
Origin of CO_2		
Via EMP–TCA	G (eq. [5])	89
Via C_1–C_6 cleavage	S (eq. [4])	11
Utilization of glucose		
Via EMP–TCA	G_e (eq. [2])	77
Via C_1–C_6 cleavage	G_p (eq. [1])	23

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INITIATION OF THE RHIZOSPHERE EFFECT[1]

J. W. ROUATT

Abstract

Microbiological analyses were conducted on unsterilized ground wheat seed, on the emerging primary root, 3 days following planting in soil and at 3-day intervals thereafter for a period of 17 days. By the third day following planting there were more than twice as many bacteria in the rhizosphere soil as in the control soil. Qualitative differences were apparent at this time also in that methylene-blue-reducing bacteria, ammonifiers, denitrifiers, gelatin-liquefying and starch-hydrolyzing types were preferentially stimulated. Studies with individual isolates obtained from samples taken even on the third day showed that rhizosphere population was more active than the soil population in regard to growth rate in different media. All effects mentioned were maintained and in some cases exaggerated with growth of the plant.

Introduction

Although a number of studies have been made on the influence of age of plant on the microflora of the rhizosphere (1, 3), only a few have attempted to demonstrate precisely at which stage in the development of the plant the rhizosphere effect manifests itself. Another point of interest concerns the contribution of the microflora of the seed to the rhizosphere microflora. Using bacterial counts as a criterion Timonin (11) showed that a rhizosphere population was established within 3 days of seed germination in soil. However, since he used surface-sterilized seed it was not possible to assess the contribution of the seed microflora to the rhizosphere population. Rovira (9), on the other hand, used unsterilized seed but grew the plants in sterile beads or washed sand, thereby completely eliminating the soil microflora. He observed an extensive development of the seed microflora as the seed germinated, which continued on the developing root. This, however, is not necessarily a true picture of the development of the rhizosphere population since the soil flora was not involved. Wallace and Lochhead (12) postulated that "the rhizosphere organisms constitute a group morphologically, physiologically, and nutritionally intermediate between the indigenous soil bacteria and the epiphytic seed microflora." This conclusion is also supported by the results of Pantos (6), who claimed that "the main forms of the rhizosphere bacteria of wheat can already be found on the seed itself." Wallace and Lochhead analyzed seed only, whereas Pantos simply made bacterial counts of the rhizosphere of the plants at different stages of development and bacterial counts of a separate lot of seed. In no instance, however, was a thorough analysis made of the changes in the microflora starting with the seed and continuing into the seedling stage. The results of such a study are presented below.

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Experimental Methods

A 10-g sample of wheat seed in 90 ml sterile water was comminuted for 3 minutes in a Waring blendor. Suitable dilutions of the resultant mixture were made and 1-ml aliquots of each were added in triplicate to tubes of appropriate media. The tubes were incubated at 26° C and examined at regular intervals. Studies on bacterial isolates were made by plating appropriate dilutions of the homogenate with soil extract agar containing 0.02% K_2HPO_4 and isolating all colonies on a suitable plate or representative section thereof following an incubation period of 14 days at 26° C. Colonies were picked and stab cultures made into soil extract semisolid agar (4) to serve as stock cultures for further study.

A portion of the same seed lot was planted in a fertile field soil in the greenhouse. Root samples for analysis were obtained, as previously described (4, 8), at 3-, 7-, 10-, 14-, and 17-day intervals after planting. Control soil samples were taken only at intervals of 3, 10, and 17 days. Dilutions of these soils were made in the usual manner and aliquots tested as above.

The following media were used: soil extract agar for bacteria counts and rose bengal – aureomycin agar for counts of fungi (7); for the physiological tests a basal medium composed of soil extract, inorganic salts, 0.1% glucose, and 0.25% yeast extract and peptone was used; for the dye-reduction test methylene blue was added to this medium to give a final concentration of 1: 200,000. Acid and gas production were determined with Durham tubes in the above medium containing bromocresol purple at a final concentration of 0.0008%. Ammonia production, aerobic cellulose decomposition, and denitrifying ability were determined as previously described (2). Starch and gelatin hydrolysis were tested in the basal medium containing 1.5% agar and either 0.2% starch or 10% gelatin.

The nutritional grouping of the isolates was carried out by the procedure previously described (5, 10) involving growth response in three media:

medium B, basal medium;
medium A, basal medium + amino acids;
medium YS, basal medium + yeast extract + soil extract.

This made possible a determination of the relative incidence of various nutritional types in the samples as well as an analysis of the amount of growth produced by all cultures in the three different media under identical conditions.

Results and Discussion

Bacterial and fungal counts and rhizosphere:soil ratios at the different sampling periods are presented in Table I. From a count of 100,000 per gram of seed, numbers rose sharply on the primary root resulting in a rhizosphere: soil (R:S) ratio of 2 for bacteria 3 days after planting. After a further increase in numbers to a R:S ratio of 5 at 10 days there appeared to be little change for the remainder of the experiment. Fungal counts showed little change at 3 days, but at 7 days there were over twice as many in the rhizosphere, an effect which persisted thereafter.

TABLE I

Numbers of bacteria and fungi on wheat seed and in rhizosphere soil at different stages of development

Sampling period, days	Bacteria, millions	R/S*	Fungi, thousands	R/S*
0 (seed)	0.1	—	3	—
3	167	2.3	77	0.7
7	345	4.7	241	2.4
10	366	5.0	257	2.5
14	362	5.0	278	2.7
17	345	4.7	224	2.2
Control soil†	73		101	

*Numbers per gram oven-dry rhizosphere soil.
Numbers per gram oven-dry control soil
†Average of numbers obtained at three sampling periods (3, 10, and 17 days).

The incidence of certain physiological groups of bacteria, calculated on the basis of most probable numbers, at the different sampling periods is presented in Table II. Root data are again presented as rhizosphere: soil ratios. A rhizosphere effect was noted in most cases at 3 days and this increased with time. Thus, even at 3 days, there were approximately 3 times as many methylene-blue-reducing bacteria, 15 times as many ammonifiers, 9 times as many denitrifiers, 10 times as many gelatin-liquefying and starch-hydrolyzing types, and 35 times as many cellulose decomposers in the rhizosphere soil as in the control soil. Where the rhizosphere: soil ratio is greater than that obtained by plate count (see Table I) a selective effect of the root is considered to have taken place. Thus, ammonifying bacteria show a R: S ratio of 15 at 3 days as compared with 2.3 for the bacterial count. The other groups indicated show a similar trend with the exception of bacteria producing acid and gas from glucose in which case the change in the R: S ratio is very similar to that shown by the bacterial population as a whole. Another point of interest is that few if any denitrifying, gelatin-liquefying, or cellulose-decomposing types were found on the seed, suggesting that the soil was the primary source of these organisms developing on the root.

TABLE II

Influence of age of plant on various physiological groups of bacteria

Physiological group	Seed* count, thousands	Control† soil, millions	R/S, ‡ days				
			3	7	10	14	17
Reducing methylene blue	25	10	2.5	9.5	9.5	12.5	15.0
Producing acid from glucose	250	5	1.0	3.0	4.0	5.0	4.0
Producing gas from glucose	—	0.15	1.0	1.5	5.0	5.0	1.3
Ammonifying	7	1.7	15.0	26.0	44.0	56.0	56.0
Denitrifying	—	0.6	9.0	9.0	9.0	19.0	50.0
Gelatin liquefying	—	0.025	10.0	10.0	18.0	38.0	38.0
Starch hydrolyzing	0.4	0.025	10.0	38.0	100.0	100.0	180.0
Cellulose decomposing	—	1.3	35.0	35.0	35.0	73.0	35.0

*Average of five samples.
†Average of numbers obtained at three sampling periods (3, 10, and 17 days).
‡Numbers per gram oven-dry rhizosphere soil.
Numbers per gram oven-dry control soil

The nutritional grouping of the isolates from seed, soil, and rhizosphere after growth in three media of increasing nutritional complexity is presented in Table III. The proportion of amino-acid-requiring bacteria from seed and soil was similar, but even at 3 days following planting there was a marked increase in the incidence of this group on the roots. A corresponding decrease in the percentage of organisms requiring vitamins and the more complex substances in yeast and soil extracts was observed. These observations are in line with previous reports from this laboratory (3, 8).

TABLE III
Percentage incidence of nutritional groups

Nutritional group	Seed*	Soil†	Rhizosphere, days				
			3	7	10	14	17
B (inorganic medium)	2	3	6	6	2	2	3
A (B + amino acids)	16	19	43	51	66	46	47
YS (B + yeast and soil extracts)	82	78	50	43	32	51	50

*Average of five samples.
†Average of three samples.

The growth rate of the isolates in the three media of increasing complexity is compared in Table IV. There was again observed a shift towards more rapidly or more profusely growing types in the rhizosphere even on the youngest roots. Thus, at 3 days in medium B, 15% of the soil isolates showed better than 90% light transmission as compared with 27% of the rhizosphere isolates; for medium A the figures are 26 and 51%, respectively, and for medium Y, 67 and 87% respectively. This shift persisted for the remainder of the experimental period.

TABLE IV
Growth of isolates in nutritional media*

Nutritional medium	Seed†	Soil†	Rhizosphere, days				
			3	7	10	14	17
B (inorganic medium)	5	15	27	35	25	18	20
A (B + amino acids)	22	26	51	57	47	58	67
YS (B + yeast and soil extracts)	64	67	87	89	84	88	94

*Expressed as per cent showing less than 90% light transmission in Luxtrol photoelectric colorimeter.
†Average of three samples.

These results indicate that even in the initial stages of its development the plant root exerts a profound effect on the numbers and types of microorganisms in the soil around it. The resulting rhizosphere microflora is undoubtedly derived from both the seed and the soil, although considering the relatively low numbers on the seed it might be justifiable to conclude that most of these rhizosphere organisms originate in the soil. They also undoubtedly owe their activity to the substances elaborated by the seed and young root. Such substances as amino acids and sugars are particluarly abundant in germinating seed and may be readily demonstrated in the medium surrounding them.

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MUCIN AS AN AGENT PROMOTING INFECTION BY PSEUDOMONAS AERUGINOSA (SCHROETER) MIGULA IN GRASSHOPPERS[1]

JUNE M. STEPHENS[2]

Abstract

The LD_{50} of *Pseudomonas aeruginosa* (Schroeter) Migula was significantly decreased when the bacteria were injected into or fed to grasshoppers along with 1% granular mucin. In feeding experiments on 490 treated and 490 control insects *P. aeruginosa* in mucin was 6.4 times (5% fiducial limits of 3.4 and 11.9) as potent as *P. aeruginosa* alone. Mucin did not appear to speed the death of the grasshoppers. The action by which mucin promotes the infection was not determined.

Introduction

Routine experiments (12) to test protective qualities of various solutions for *Pseudomonas aeruginosa* (Schroeter) Migula showed that the pathogenicity of the bacteria for grasshoppers was increased when the organisms were suspended in granular mucin (type 1701-W, Wilson Laboratories, Chicago, Illinois). The experiments reported herein chiefly concern a comparison of median lethal doses in grasshoppers after they were inoculated with or fed the bacteria along with granular mucin as opposed to controls inoculated with or fed *P. aeruginosa* alone.

Experimental infections of laboratory mammals by several bacterial species have been promoted by the use of mucin (1, 9, 11). So far as the author is aware, this is the first report of use of mucin to enhance an infection in insects. Infection of mammals by bacteria has frequently been promoted by parenteral injection of mucin before or along with injection of the bacteria. This is a report on effects of mucin administered orally, as well as by injection, in promoting infection by *P. aeruginosa* in grasshoppers.

Preparation of Mucin Suspensions, Cultures, and Diluting Fluid

A 1% suspension of mucin in Bacto nutrient broth (Difco Laboratories, Incorporated, Detroit, Michigan) was most satisfactory for this investigation. It was prepared by adding 1.0 g of the granular mucin to a portion of 100 ml of unsterilized Bacto nutrient broth at pH 7.4. The sticky mass was rubbed relatively free of lumps with a glass rod and the remainder of the broth added. The pH of the suspension dropped from 7.4 to about 6.7. Flasks containing 100 ml of the mucin–broth suspensions were autoclaved at 15 lb for 15 minutes. The pH was adjusted to 7.4 with $N/10$ NaOH after the suspension cooled. Though the manufacturer's directions stated that the pH should not be adjusted before the suspension was sterilized, the pH may be adjusted before or after autoclaving without affecting the ability of the mucin to

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promote infections by *P. aeruginosa* in grasshoppers. Inoculation of the mucin–broth with a strain of *P. aeruginosa* isolated from grasshoppers was made in the normal manner. All cultures were grown for 20 hours at 37° C.

The bacterial cultures were diluted with a 1% aqueous solution of mucin prepared as described above. As some insoluble particles were present in the suspension, only the supernatant was used so as not to occlude the No. 30 hypodermic needles used to inoculate the grasshoppers.

Tests for Toxicity of Mucin to Grasshoppers

Tests were made to determine whether the mucin alone was toxic to grasshoppers. Four groups each of 10 adult grasshoppers, *Melanoplus bivittatus* (Say), were injected with 0.01 ml of solutions containing 0.0001, 0.001, 0.01, and 0.1 mg of mucin, and observed during 7 days with an equivalent number of uninjected controls. In the main experiments a grasshopper receiving 0.01 ml of a dilution of *P. aeruginosa* grown in 1% mucin–broth received 0.0001 mg of mucin. There were equal mortalities (3%) in test and control insects, indicating that mucin apparently has no toxic effect upon grasshoppers in the quantities injected. Grasshoppers individually fed lettuce contaminated with 0.1 mg of mucin showed no ill effects.

Inoculation and Feeding Experiments

Forty grasshoppers were each inoculated with 0.01 ml of 1% mucin and 4 hours later were divided into groups of 10 and injected with various doses of *P. aeruginosa*; 40 more grasshoppers were similarly injected with *P. aeruginosa* culture but were not previously injected with mucin. Two additional groups of 40 grasshoppers each were injected with the bacteria in mucin suspension, two control groups being injected with *P. aeruginosa* culture only. The insects were observed for 1 week.

In the feeding tests, all grasshoppers were first starved for 1 day. All treated grasshoppers were individually fed lettuce contaminated with *P. aeruginosa* suspended in mucin–broth and the controls were similarly fed *P. aeruginosa* alone. Seven doses of increasing size were each fed to lots of 10 grasshoppers in treated and control groups and the experiments were repeated seven times. The insects were observed for 30 days. As previous work (13) showed that maintaining the grasshoppers in vials contributed to infection, all grasshoppers were individually maintained in half-pint cardboard containers.

The results of each experiment were analyzed by the method of Litchfield and Wilcoxon (6), to estimate the LD_{50} and the relative potency, with its fiducial limits. The relative potencies were tested for departure from homogeneity by a chi-square test, and were combined to give mean values by the weighting method outlined by Finney (3).

When mucin was injected before the bacteria, the LD_{50} of *P. aeruginosa* was 5.6 and that of controls was 31, the potency ratio of 5.5 being significant at the 5% level. When the bacteria were injected in a suspension of 1%

mucin, mucin increased the potency of the culture 5.8 times with 5% fiducial limits of 2.0 and 17.0 (Table I). Further inoculation experiments were not attempted; *P. aeruginosa* is relatively pathogenic to grasshoppers by inoculation so it might sometimes be difficult to show significant reductions in LD_{50}. Mucin evidently has the same infection-promoting effect for *P. aeruginosa* whether injected along with or several hours before the bacteria.

TABLE I

Estimated ratios of potencies, for grasshoppers, of *P. aeruginosa* injected alone and in mucin suspension

Experiment No.	LD_{50} Without mucin	LD_{50} With mucin	Potency ratio*
1	22.0 (7.3, 66.0)†	3.80 (1.3, 11.0)	5.8‡
2	41.0 (15.2, 110.7)	6.70 (2.2, 20.1)	6.1‡

*Weighted mean 5.8, with 5% fiducial limits 2.0 and 17.0.
†5% fiducial limits.
‡Significant at 5% level.

In the seven feeding experiments, six of the seven potency ratios differed significantly from unity at the 5% level (Table II). Though the potency ratios varied appreciably between experiments, the variation was not significantly greater than that within experiments. Mucin increased the mean potency 6.4 times, with 5% fiducial limits 3.4 and 11.9.

TABLE II

Estimated ratios of potencies, for grasshoppers, of *P. aeruginosa* fed alone or in mucin suspension

Experiment No.	LD_{50} ($\times 10^{-2}$) Without mucin	LD_{50} ($\times 10^{-2}$) With mucin	Potency ratio*
1	80 (31, 208)†	23 (9, 59)	3.5
2	1170 (468, 2925)	255 (106, 612)	4.6‡
3	350 (93, 1323)	37 (10, 127)	9.6‡
4	250 (64, 975)	21 (7, 59)	12.20§
5	87 (15, 497)	7 (2, 24)	12.3‡
6	375 (95, 1470)	52 (16, 174)	7.2‡
7	13 (4, 41)	2 (0.5, 8)	6.8‡

*Weighted mean 6.4, with 5% fiducial limits 3.4 and 11.9.
†5% fiducial limits.
‡Significant at 5% level.
§Significant at 1% level.

Though mucin promoted the effect of *P. aeruginosa*, it did not appear to have any effect on the speed of death of the grasshoppers, as 55% of the total mortality in both the insects treated with mucin and in those fed *P. aeruginosa* alone had occurred by the 15th day.

Discussion

It is noteworthy that, as in mammals, mucin promotes infection by inoculated doses of *P. aeruginosa* in the grasshopper. More interesting is the

finding that it also significantly decreases the LD_{50} when the bacteria are fed along with it; evidently mucin exerts its virulence-enhancing effect on mammals only when it is administered by the parenteral route.

As in most biological tests of this nature, the differences in LD_{50} from one experiment to another are possibly due to variation in the populations. However, experimental conditions for the feeding experiments were not ideal as it was never possible to be certain that a grasshopper would eat an infected piece of lettuce before some of the bacteria were knocked from it in the feeding vial. Preliminary experiments showed that force-feeding was not satisfactory, as the grasshopper usually regurgitated immediately after feeding so that much of the culture was lost.

As for grasshoppers, mucin is effective as a virulence-enhancing agent in mammals when injected before the bacteria (1). Because of the considerable variations in times of feeding it was not practical to test the action of mucin by ingestion several hours before ingestion of the bacteria.

A luxuriant growth of *P. aeruginosa* was always obtained in mucin–broth. MacCabe and King (8) stated that mucin stimulated the growth of some bacteria markedly, and speculated on whether a similar effect occurred in the animal body. The possibility of greatly increased growth in the grasshopper body after ingestion of the bacteria seems doubtful in view of the length and variation in time of infection in the grasshopper.

The action by which mucin promotes infection by *P. aeruginosa* in grasshoppers is as yet undetermined. There are many theories as to the manner in which it exerts its effect in mammals. Anderson and Oag (1) stated that mucin does not promote infection by all types of bacteria. Nungester *et al.* (10) stated that mucin could not lend virulence to nonvirulent cultures whereas Ercoli *et al.* (2) stated that it decreased the resistance of the host to bacteria even when their invasive power was low. *P. aeruginosa* is relatively pathogenic for the grasshopper, and the action of mucin for nonpathogens of insects remains to be investigated.

Lambert and Richley (5) investigated the action of mucin in promoting infection by several species of bacteria in mice. They stated that heparin is the active component of mucin and that it interferes with complementary and opsonic action, thus contributing to depression of bacteriolysis and inhibition of phagocytosis. Other workers (7, 9) postulated inhibition of phagocytosis by coating of bacteria or damage to phagocytes; Olitzki (11), in a review on the action of mucin, concluded that most of the effects probably resulted from a coating action on the bacteria. Miller and Castles (9) stated that mucin did not enhance the virulence of meningococci in the conventional sense, as organisms recovered from infected mice were no more virulent than those grown on artificial media; they contended that the lethal dose was greatly lowered when the bacteria were suspended in mucin before intraperitoneal injection and that the apparent enhancement of invasiveness seemed to be caused by local interference with the defense mechanism of the host. Keefer and Spink (4) suggested that in some way mucin protected the bacteria from the action of antibody, thus permitting bacterial growth.

Though little is known about the immune reactions of grasshoppers, it appears that they do not form antibodies in the conventional sense (14) and, therefore, the action of mucin in promoting infection by *P. aeruginosa* in grasshoppers is probably not by inhibition of a true antibody reaction. However, the possibility exists that, as in mammals, mucin may interfere with the defense mechanism of the grasshopper. Further studies on the immune reactions of insects may give valuable information as to the action by which mucin aids the invasiveness of bacteria for insects.

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METABOLIC ACTIVITY AND PHOSPHATE-DISSOLVING CAPABILITY OF BACTERIAL ISOLATES FROM WHEAT ROOTS, RHIZOSPHERE, AND NON-RHIZOSPHERE SOIL[1]

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Abstract

Bacterial isolates from the roots of wheat (rhizoplane) were more active in oxidizing glucose and alanine than cultures isolated from rhizosphere and non-rhizosphere soils. In general, metabolic activity was greater with alanine than with glucose. Over one third of the cultures tested were capable of dissolving insoluble phosphorus in the form of $CaHPO_4$ but the roots did not appear to exert a selective effect on these forms. However, the phosphate-solubilizing organisms from the rhizoplane were also the most active in oxidizing glucose and alanine. Those from the rhizosphere soil were intermediate in this respect. By far the majority of these phosphate-dissolving bacteria were in the nutritional group requiring unknown substances in yeast and soil extracts for optimal growth. It was suggested that although these bacteria were not preferentially stimulated in the root zone, their large numbers and their greater metabolic activity may contribute significantly to the phosphate economy of the plant.

It was reported recently (12) that bacterial isolates from the soil in the vicinity of wheat roots (the rhizosphere) were more active in oxidizing various substrates than those from soil a short distance away from the roots. These studies have now been extended to include bacteria more intimately associated with the root surface (the rhizoplane) (1) than those in the rhizosphere soil itself. The purpose of this investigation was to determine if rhizoplane bacteria differed appreciably from those in the rhizosphere soil by virtue of their intimate relationship with the root and whether the rhizosphere effect was intensified on the root surface. The present paper is concerned with the metabolic activity of these bacteria; their nutritional requirements and general physiological behavior as well as the over-all rhizosphere effect will be reported in a subsequent publication.

At the same time, it was thought desirable to determine if any relationship could be established between the metabolic activity of the bacterial isolates used and their ability to dissolve precipitated phosphate in the form of $CaHPO_4$. It has been reported by Rose (7) and Sperber (10) that phosphate solubilization by microorganisms is related to acid production which is, of course, intimately related to metabolism. Sperber (10) has also stated that a "higher percentage of phosphate-solubilizing bacteria were isolated from the rhizosphere of plants." The interesting work of Gerretsen in 1948 (3) demonstrates strikingly the influence of bacteria in association with roots in rendering phosphates soluble and the recent work of Russian investigators

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[2]National Research Council of Canada Fellow in collaboration with the Canada Department of Agriculture, 1957–1959.

appears to support this observation (9, 11). However, there is comparatively little evidence in the literature of a preferential stimulation of phosphate-dissolving bacteria in the rhizosphere. The tests reported below were designed to yield more information on this subject also.

Materials and Methods

The method of isolating bacteria from rhizosphere and non-rhizosphere soil has been described previously (5). For isolation from the root surface or rhizoplane, the root material used to provide the rhizosphere soil sample was washed in three changes of sterile water, resuspended in 100 ml sterile water, and macerated in a Waring Blendor under aseptic conditions for 3 minutes (8). Serial dilutions of this homogenate were prepared and aliquots plated out in the usual manner. All cultures were maintained in soil extract semisolid agar.

For metabolic studies transfers were made to slants of Penassay base agar (Difco) containing 1% glucose and made up with soil extract. After a suitable incubation period the cells were suspended in a sterile solution containing 0.5% yeast extract and the suspension used to inoculate Roux bottles containing the agar medium. After 48 hours' incubation at 26° C (for slowly growing forms 72 to 96 hours) the cells were harvested, washed, and resuspended in $M/20$ phosphate buffer to give a reading of 600 on a Klett–Summerson colorimeter with a 600 mμ filter. Two milliliters of this suspension were pipetted into Warburg vessels containing 0.2 ml 20% KOH in the center well and 0.1 ml $M/20$ substrate in the side arm. After a 30-minute equilibration period the substrate was tipped into the main compartment and oxygen uptake determined at 15-minute intervals for 2 hours at 30° C. All results are reported with the endogenous values subtracted.

To determine the ability of the isolates to dissolve inorganic phosphate ($CaHPO_4$) two media were used: *medium 1* was soil extract agar containing 1% glucose and 2% agar; *medium 2* consisted of glucose 1%, agar 2%, asparagine 0.2%, $MgSO_4$ 0.05%, NaCl and KCl 0.01% each, yeast extract 0.05%, and tap water 1 liter. The media were dispensed in 100-ml amounts for sterilization. The sterile media were cooled to 50° C and 5 ml sterile 10% K_2HPO_4 and 10 ml sterile 10% $CaCl_2$ were added to each flask to produce a fine precipitate of $CaHPO_4$. The reaction of the medium was then adjusted to pH 7.0 with sterile $N/1$ NaOH and plates poured immediately. Loopfuls of cells from fresh yeast beef agar cultures were placed on the plates, five per plate, and the plates incubated at 28° C. Zones of clearing around the colonies were recorded after 7 days.

Experimental Results

The results of the manometric experiments with isolates from the rhizoplane and also from rhizosphere and non-rhizosphere soil are summarized in Table I. With either glucose or alanine as substrates the mean metabolic activity is greatest with cultures from the rhizoplane, those from the rhizosphere soil

being intermediate. It is noteworthy that the values for alanine are higher in every instance than those for glucose indicating the importance of this type of material for these soil organisms. An interesting anomaly in these results may be observed on comparing the endogenous values of the three groups of cultures, that for the rhizoplane organisms being markedly lower than those for rhizosphere and control soil organisms.

TABLE I

Oxygen uptake (μl) of bacterial isolates from rhizoplane of wheat and from rhizosphere and non-rhizosphere soil (after 2 hours)

Sources of cultures	Number tested	Average endogenous values	Average glucose values (−endog.)	Average alanine values (−endog.)
Rhizoplane	49	59	234	306
Rhizosphere soil	56	206	198	247
Control soil	68	131	113	141

The distribution of oxygen-uptake values for the individual cultures is given in Figs. 1 to 3 which also show that most of the rhizoplane cultures are more active on the substrates used than the rhizosphere soil cultures and the latter are, in general, more active than those from the control soil. The endogenous values of the rhizoplane cultures are again the outstanding exception to this general trend.

The phosphate-dissolving capacity of these and additional cultures on the two media tested is given in Table II and illustrated in Fig. 4. There were no appreciable differences between the two media nor were there any significant differences in the proportion of organisms capable of dissolving insoluble phosphate in the three groups of isolates tested. A comparison of phosphate-dissolving capacity with metabolic activity, as measured by oxygen uptake,

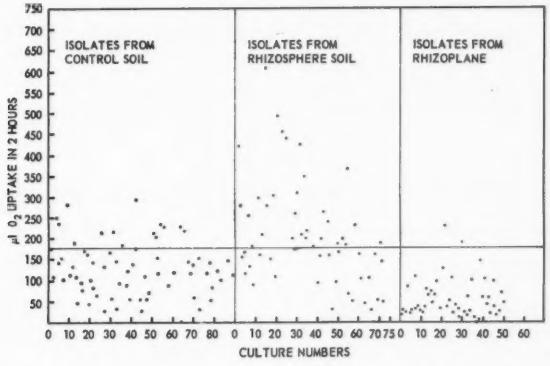

FIG. 1. Oxygen uptake in absence of substrate by bacterial cultures from the rhizoplane of wheat and from rhizosphere and control soil.

showed that although most of the phosphate-dissolving bacteria from the rhizoplane were very active on both glucose and alanine, most of those in the control soil were relatively inactive (Table III). The isolates from rhizosphere soil were again intermediate in this respect.

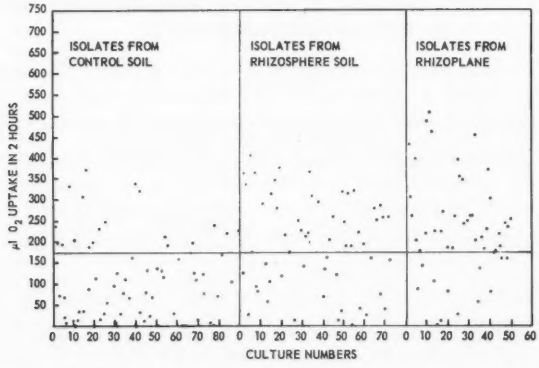

FIG. 2. Oxygen uptake with glucose as substrate by bacterial cultures from the rhizoplane of wheat and from rhizosphere and control soil (endogenous values subtracted).

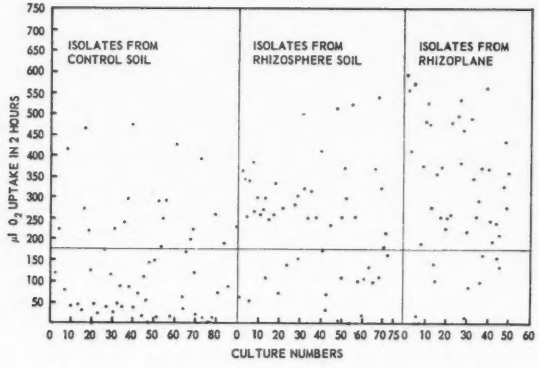

FIG. 3. Oxygen uptake with alanine as substrate by bacterial cultures from the rhizoplane of wheat and from rhizosphere and control soil (endogenous values subtracted).

TABLE II

Phosphate-dissolving capacity of cultures from rhizoplane and from rhizosphere and control soil

Source of cultures	Number tested	Phosphate-dissolving cultures, %	
		Medium 1 (soil extract)	Medium 2 (semisynthetic)
Rhizoplane	90	36	35
Rhizosphere soil	92	35	36
Control soil	94	39	45

PLATE I

FIG. 4. Dissolution of phosphate as $CaHPO_4$ in soil extract agar containing glucose by bacterial isolates from soil.

Since the nutritional requirements of all these organisms had been studied (8) it was possible to determine the distribution of the phosphate-dissolving organisms among the nutritional groups to which they were assigned. The results of this comparison are shown in Table IV. Although an appreciable number of amino acid requiring bacteria from the rhizoplane are capable of dissolving phosphate, the most striking feature of this comparison is the large proportion of phosphate-dissolving organisms in the group which requires substances present in yeast and soil extracts for growth.

TABLE III

Relationship between phosphate-dissolving capacity and metabolic activity*

Source of cultures	Endogenous	Glucose	Alanine
Rhizoplane	0	75	86
Rhizosphere soil	67	53	47
Control soil	27	31	38

*Estimated as percentage of phosphate-dissolving cultures consuming over 175 μl oxygen in 2 hours.

TABLE IV

Distribution of phosphate-dissolving bacteria in relation to their nutritional requirements (%)

Source of cultures	B (inorganic)	A (amino acids)	G (vitamins)	YS (yeast and soil extracts)
Rhizoplane	0	30	10	60
Rhizosphere soil	0	18	6	77
Control soil	2	5	13	80

Discussion

The observed increase in metabolic activity of bacteria from the root surface or rhizoplane indicates that the plant root exerts a strong selective action favoring organisms which are physiologically more active than those in the soil away from the root. The bacteria in the rhizosphere soil occupy an intermediate position in this respect as shown also by nutritional and growth rate studies and other physiological tests to be reported in a subsequent paper (8). It is of interest that greater activity is shown by most of the cultures tested on an amino acid rather than on a carbohydrate substrate, suggesting the importance of the former group of substances for soil microorganisms. The greatest activity in this connection was shown by the rhizoplane forms with a difference between the alanine and glucose values of 72 μl of oxygen consumed. These results are suggestive of a greater availability of amino acids than of carbohydrates on the root surface and of a bacterial population adapted to these nitrogenous compounds.

The major metabolic difference between the rhizoplane bacteria and those from the rhizosphere and control soil is the low endogenous activity of the former despite their generally greater activity on glucose and alanine. Conceivably these very actively metabolizing forms do not store reserve materials such as polysaccharides and related compounds and, therefore, do not have

a supply of endogenous substrate to use. It is tempting to suggest that these rhizoplane organisms are quite different from the remaining types and may constitute a bacteriorrhizal development somewhat analogous to mycorrhiza (4).

The observation of Sperber (10) that plants exert a selective effect on phosphate-solubilizing bacteria has not been confirmed with wheat. However, because of the greater metabolic activity of rhizoplane and rhizosphere cultures it is quite conceivable that a greater phosphate turnover occurs at the root–soil interface. An additional consideration is the fact that although the proportion of phosphate-dissolving bacteria is not changed in the root zone, the total number of these organisms is much greater. The numbers of bacteria in the regions tested, as determined by plate counts with soil extract agar, were 86 and 655 millions per gram oven-dry soil in control and rhizosphere soil, respectively, and 2300 millions per gram dry root material in the rhizoplane. The total number of bacteria capable of acting on insoluble phosphate may then be calculated from the distribution data in Table II: 33.7 and 187 millions per gram oven-dry soil for control and rhizosphere samples, respectively, and 640 millions per gram dry root material for the rhizoplane. Thus there are approximately 6 times as many of these bacteria in the rhizosphere soil and about 18 times as many in the rhizoplane as in the control soil. These facts combined with the evidence of greater metabolic activity of the bacteria in the rhizoplane and the rhizosphere may be significant in relation to the phosphate economy of the plant.

Another striking observation deriving from the tests on phosphate dissolution is the high percentage of these bacteria in the nutritional group requiring yeast and soil extracts for optimum growth. Lochhead and Chase (6) have pointed out that this group includes predominantly pleomorphic forms such as "soil corynebacteria" or *Arthrobacter* whose function has not yet been defined clearly. Their ability to dissolve phosphate suggests at least one role of these bacteria in soil.

The importance of microbial dissolution of either inorganic or organic phosphate is difficult to assess in a zone such as the rhizosphere which is teeming with microbial life, since competition for phosphorus as well as for other elements must of necessity be intense. The results of Gerretsen (3) support the thesis that microorganisms can make insoluble phosphorus available to plants and Uarova (11) reports increased yields of millet and oats after inoculation with phosphate-dissolving bacteria. On the other hand Dikusar (2) reports decreased uptake by plants of mineral phosphorus, methionine, and vitamin B_1 as a result of microbial action. The phosphate ion, methionine, and vitamin were apparently converted to organic compounds largely unavailable to plants. Ribonucleic acid, which was also tested, was only slowly decomposed by microorganisms and the uptake of its decomposition products was only 6% of that of mineral phosphorus. It appears, therefore, that considerably more work is required to establish with certainty how much benefit, if any, the plant derives from the microbial mantle on its roots.

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A METHANOL-UTILIZING BACTERIUM

I. DESCRIPTION AND NUTRITIONAL REQUIREMENTS[1]

T. KANEDA[2] AND J. M. ROXBURGH

Abstract

A *Pseudomonas* isolated from soil, capable of growing on a medium containing methanol as the only carbon source, is described. Biotin or one of a few closely related compounds is required in very low concentrations as a growth factor. It is shown that the ability to use methanol is an adaptive system and that the biotin is incorporated in the cell, but is recoverable on acid hydrolysis.

Introduction

Interest in the metabolism of methanol arises primarily from its toxicity for humans and from its role as the source of the labile methyl group in biological systems (11). In wine, the spontaneous hydrolysis of methyl esters of pectic acids is regarded as the source of methanol (2), but the prior source of this methoxyl group is not known. In addition, some organisms are capable of producing methane and carbon dioxide from methanol (7, 8).

Some years ago an attempt was made to isolate microorganisms capable of utilizing methanol as a sole carbon source. A few were isolated, but all required growth factors and eventually growth became poor with all strains. Recently a *Pseudomonas* sp., PRL-W4, has been isolated which requires only biotin or one of its analogues as an essential growth factor on any medium tested. The isolation, morphology, and physiological characteristics of this organism are described in this paper.

Materials and Methods

Culture

The strain was maintained on a medium containing 5 ml of methanol, 1 μg of biotin, inorganic salts, and 15 g of washed agar per liter. The levels of inorganic salts used were the minimum concentrations found to maintain maximal growth of the organism. Unless otherwise noted all media contained per liter: potassium phosphate (dibasic), 2.0 g; ammonium sulphate, 2.0 g; magnesium sulphate (heptahydrate), 0.025 g; sodium chloride, 0.5 g; ferrous sulphate (heptahydrate), a trace; and distilled water. Other nitrogen sources were tested but none gave growth superior to that obtained with ammonium sulphate. Potassium nitrate (1 g per liter) and urea (0.1 g per liter) were satisfactory but higher concentrations of urea inhibited growth completely. Growth could not be obtained with glycine as the source of nitrogen.

[1]Manuscript received September 30, 1958.
Contribution from the Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 5033.
[2]National Research Council of Canada Postdoctorate Fellow. Present address: Department of Biochemistry, Western Reserve University, Cleveland, Ohio.

Conditions of temperature and initial pH affected the growth of PRL-W4 as indicated in Table I. The organism favors a neutral or alkaline medium and better growth is obtained at 30° C than at 25° C. At 37° C growth is completely inhibited.

TABLE I

Effect of pH and temperature on growth of PRL-W4

pH	Temperature, ° C	Cell concentration, K–S units
8.0	30	76.8
7.0	30	84.7
6.0	30	33.2
5.0	30	31.7
4.0	30	0
7.0	23	18.5
7.0	30	79.0
7.0	37	0

NOTE: Medium—Inorganic salts, 0.5% methanol, 1.0 μg per liter biotin, adjusted to specified pH with *N* potassium hydroxide; 100 ml in 500-ml flask. Culture—Six days on rotary shaker at specified temperature.

Cells were harvested by centrifugation, washed, and a suitable suspension prepared. Cell concentrations were measured in terms of Klett–Summerson (K–S) units using a Klett–Summerson colorimeter with a 500 mμ filter. This method gave a linear relation between optical density and cell concentration up to 200 K–S units. Cells for use in manometric experiments were prepared in a similar fashion but were washed with and suspended in 0.5% sodium chloride solution.

Experiments with growing cells were conducted in 250- or 500-ml flasks, containing respectively 50 or 100 ml of medium, on a rotary shaker at 30° C. Growth was not obtained in Monod's tubes, probably because of insufficient aeration. The ability of the organism to utilize methane was tested by placing the flasks in a desiccator containing an atmosphere of 50% methane and 50% air. Controls were incubated in the same fashion with methanol as the substrate and air as the gas phase. A reciprocal shaker provided the required agitation.

Reagents

Dr. R. L. Wolfe[3] kindly supplied the samples of D-homobiotin and D-norbiotin. Other biotin derivatives were obtained from Dr. W. A. Taber and diaminobutyric acid from Dr. E. Bilinsky, both of this Laboratory. Diaminopelargonic acid was prepared by du Vigneaud's method (10) and purified by preparative paper chromatography. The solvent used was the upper phase of butanol – acetic acid – water (4:1:5) and the position of the compound on the paper was detected using a ninhydrin spray on a test strip. Very dilute solutions of compounds to be tested as growth factors were kept in *M*/200 phosphate buffer, pH 7.0. Agar was washed with distilled water in a glass column for several weeks and lyophilized. Other chemicals were commercial lots.

[3]Hoffman-La Roche Co., Montreal, Que., Canada.

Respiration Measurements

Conventional Warburg cells with air as the gas phase were used for the manometric determination of respiration. One milliliter of the mixture containing the cells was used in a 5-ml flask.

Bioautography

The bioautographic technique for the location of growth factors on paper chromatograms is particularly suitable for use with this organism because of the pigmented nature of the cells. A thick layer of a medium containing 1.5% washed agar, salts, and 0.3% aspartic acid as a carbon source was placed in a covered glass tray. The paper chromatogram was left in contact with the solidified agar for several hours, removed, and the agar sprayed with a dilute suspension of washed cells of PRL-W4. After 2 days of incubation at 30° C the location of any spots containing growth factors was clearly indicated by growth of reddish colonies. Liquid medium was used in some cases by cutting the paper chromatogram in small pieces and eluting each with the medium, lacking only a growth factor. The tubes were inoculated with a washed cell suspension and incubated. Cell growth, as measured by optical density, indicated the presence of a growth factor in that part of the chromatogram.

Chromatography

Paper chromatograms were prepared on Whatman No. 2 paper and eluted with the upper phase of a freshly prepared butanol – acetic acid – water solvent (4:1:5) at room temperature. Amino acids were detected using a ninhydrin spray. Growth factors were located by the bioautographic methods described above.

Recovery of Growth Factors from Cells

Cells were acid-extracted and hydrolyzed to provide samples for chromatography and bioautography. Washed cells which had been grown either on a 0.5% methanol medium containing 1 μg of biotin per liter or on a 0.3% L-aspartate medium containing 0.1 μg of biotin per liter were refluxed with normal (N) hydrochloric acid for $\frac{1}{2}$ hour. The cells were separated by centrifugation and subjected to hydrolysis with 6 N hydrochloric acid in sealed glass tubes at 120° C for 16 hours.

Experimental and Results

Isolation

Soil samples from a number of places in Canada and Japan were used. Serial transfers were made from the initial soil suspension using a medium containing inorganic salts and 0.5% methanol. In all cases growth ceased after five transfers or less. When a transfer from the third liquid culture was made to agar plates three samples showed extensive growth. All were from rich loam soil samples, one from Saskatoon, one from Edmonton, Alberta, and one from Tokyo, Japan. The colonies developed in all three cultures appeared to be pure strains and all were reddish and of a similar appearance.

When one colony from each was transferred back to the liquid medium growth was obtained in all cases. Each isolate was purified by plating on agar.

The Tokyo strain was selected for further study since it was somewhat more vigorous although, in common with all organisms which use one-carbon compounds as sole carbon source, growth was slow as compared with other aerobic organisms.

Morphology and Staining

The Tokyo strain, designated PRL-W4, is a Gram-negative rod with rounded ends, measuring 0.5 by 1.2 microns (Fig. 1A). It occurs singly, is motile with a single flagellum, and does not form spores at any stage. Colonies after 3 days on the agar medium are pin-point, moist, raised, circular, and smooth-edged. Their diameter reaches 2 mm maximum after 1 week (Fig. 1B). The organism is strictly aerobic, and quiescent cultures develop turbidity only in the surface layer.

Pigmentation[4]

The cells of this organism contain a red pigment regardless of the carbon source on which they are grown. Some of this pigment was extracted from lyophilized cells with cold ethanol and fractionated with an ether–ethanol system. When chromatographed on a silica column using a mixture of petrol ether and methanol as the eluting solvent a single band was obtained. Absorption maxima for the eluted material were obtained at 460, 489, and 523 mμ in ethyl ether and at 455, 488, and 520 mμ in ethanol, characteristic of the more unsaturated carotenoid type of pigment. The pigment is acidic and readily extracted from ethyl ether with 60% ethanol containing 5% potassium hydroxide.

Identification of Growth Factor

It was apparent that at least one growth factor was required by PRL-W4 since growth ceased after five transfers from the original soil suspension. Likewise, although growth was obtained on commercial agar, no growth occurred when the medium was made up with thoroughly washed agar (Fig. 1C). Tests showed that the growth factor could be provided by the addition of yeast extract (which also supplied a carbon source) but not by casein hydrolyzate. A mixture of 10 vitamins added to the methanol medium stimulated growth and when these were tested individually biotin alone showed growth-promoting activity.

Biotin was tested in conjunction with each of 16 other compounds (Table II). None of these stimulated growth to any large extent, but pantothenate, uracil, and xanthine did increase the cell yield significantly.

Effect of Biotin Concentration

The initial concentration of biotin in the medium directly affected both the yield of cells and the rate of growth during the logarithmic phase (Fig. 2).

[4]We are indebted to Dr. F. W. Hougen for his assistance.

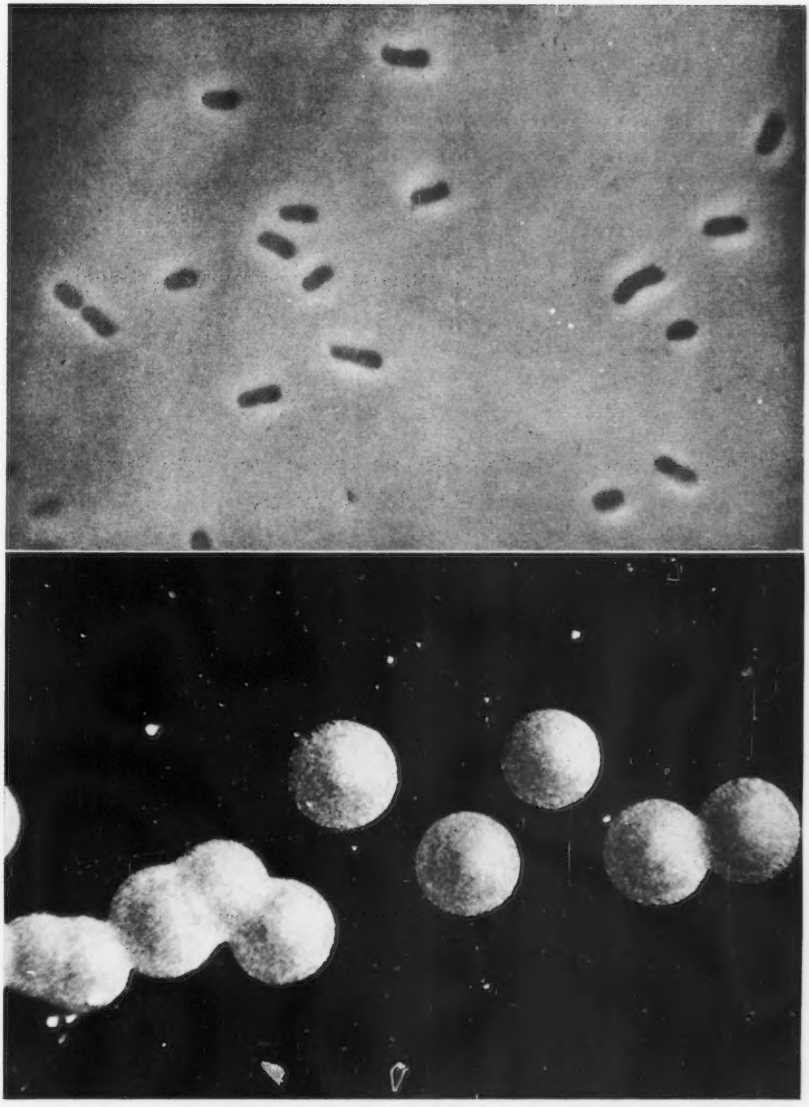

FIG. 1. Single cells and colonies of PRL-W4.
A, upper. Typical cells—dark field, phase microscope, ×2000 (approx.).
B, lower. Typical colonies—agar medium containing 0.5% methanol and 1.0 μg/liter biotin.

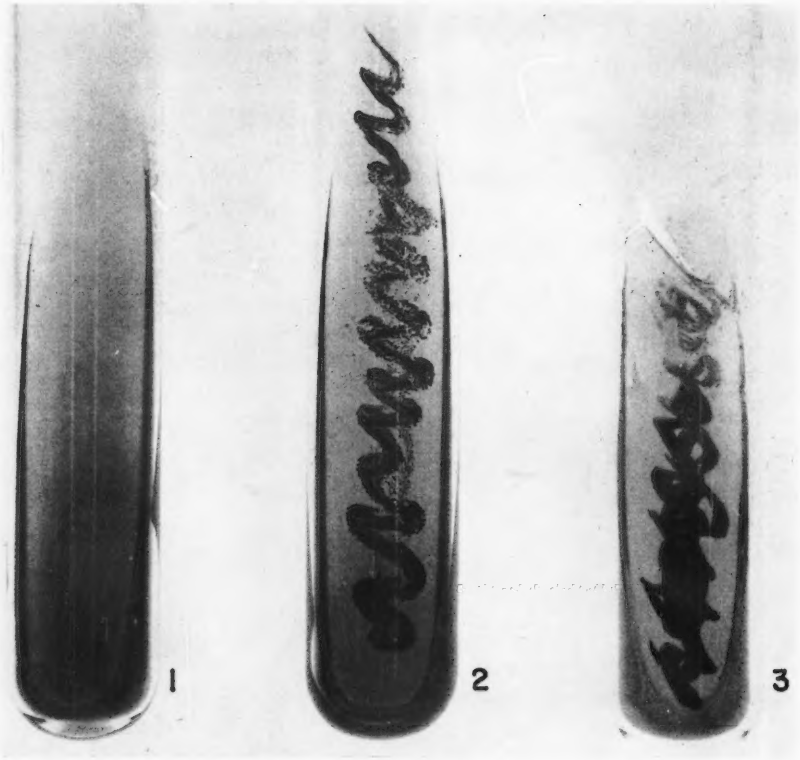

FIG. 1C. Typical agar slant cultures—washed agar:
1. No growth factor, 0.5% methanol.
2. Biotin 1 μg/liter, 0.5% methanol.
3. Yeast extract 0.1%, 0.5% methanol.

Little effect was shown at concentration greater than the 0.12 μg per liter in the methanol medium. Very low concentrations of biotin, biotin sulphoxide, or desthiobiotin were not effective in promoting growth.

TABLE II

Growth of PRL-W4 on methanol media containing vitamins with and without biotin

Vitamin added, 50 μg/l.	Growth after 4 days, Klett-Summerson units*	
	With 1 μg/l. biotin	Without biotin
None	77.3	–
Pantothenate	105.2	0
Pyridine	79.7	0
Thiamine	92.7	0
Folic acid	91.3	0
Choline	85.9	0
Nicotinic acid	94.3	0
Inositol	81.3	0
p-Aminobenzoic acid	87.4	0
Riboflavin	82.2	0
B_{12}	68.2	0
Glutathione	92.8	0
Uracil	104.8	0
Guanine	82.5	0
Xanthine	101.2	0
Adenine	87.5	0
L-Tryptophane	88.5	0
All above vitamins		0

NOTE: Medium—Inorganic salts, 0.5% methanol, vitamins, and biotin as indicated; 50 ml in 250-ml flask.
Culture—Rotary shaker at 30° C.
*See text.

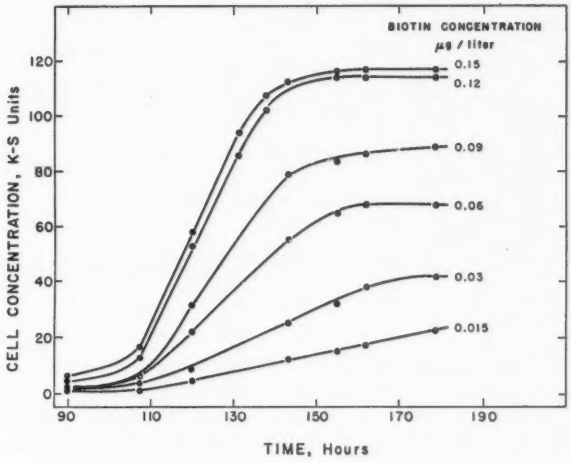

FIG. 2. Effect of biotin concentration on the growth rate and cell production of PRL-W4.
Medium: 0.5% methanol, inorganic salts, plus biotin as indicated; 50 ml in 250-ml flask.
Culture: Rotary shaker at 30° C.
Samples: Removed for optical density reading and returned aseptically.

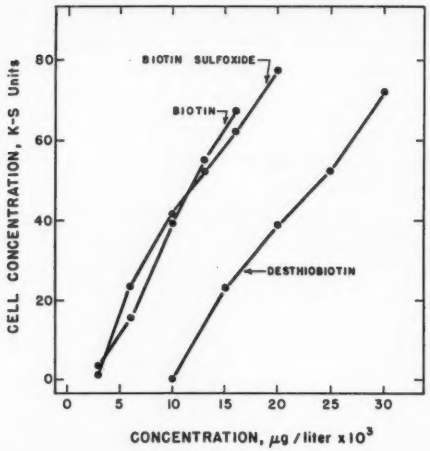

FIG. 3. Effect of biotin and biotin derivatives on the cell yield of PRL-W4.
Medium: Inorganic salts, 0.3% L-aspartate, and 1% biotin, biotin sulphoxide, or desthiobiotin as indicated; 50 ml in 250-ml flask.
Culture: Rotary shaker at 30° C.
Maximum cell growth in terms of Klett–Summerson colorimeter units is reported for each culture.

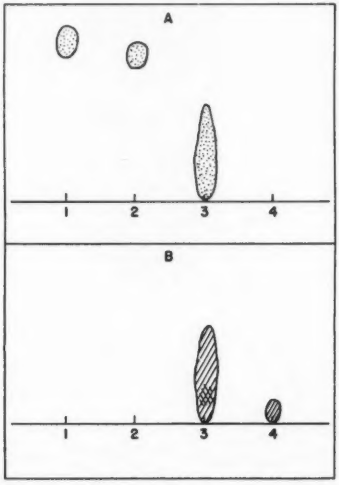

FIG. 4. Comparison of locations of ninhydrin-positive and growth-promoting spots on paper chromatograms of biotin and related compounds. Compounds chromatographed: (1) biotin, (2) desthiobiotin, (3) diaminopelargonic acid, (4) lysine.
A. Location of growth-promoting compounds as determined by bioautographic method described in text.
B. Location of ninhydrin positive compounds.

Substitutes for Biotin

Several compounds closely related to biotin were tested for activity as growth factors for PRL-W4. The standard medium, but with 0.3% aspartate as the carbon source, was used for these tests. Biotin sulphoxide is equivalent to biotin as a growth factor and desthiobiotin is also effective but only at higher concentrations (Fig. 3). Biocytin was inactive.

Diaminopelargonic acid can replace biotin as the growth factor as is shown in the bioautographs depicted in Fig. 4. Other diamino acids (ornithine, lysine, and 2,4-diaminobutyric acid) showed no activity nor did pelargonic acid, pimelic acid, or Tween 80. Pelargonic acid was also tested in the presence of urea without effect. Apparently the organism is not capable of synthesizing either desthiobiotin from pelargonic acid and urea or diaminopelargonic acid from pelargonic acid and ammonium ion.

Norbiotin and homobiotin act as competitive inhibitors with some fungi and bacteria that require biotin as a growth factor (1, 4, 9). PRL-W4 was not inhibited but rather slightly stimulated by the addition of these compounds to the medium containing biotin (Table III). At a concentration of 10 μg per liter norbiotin showed some activity as a growth factor in the absence of biotin.

TABLE III

Effect of norbiotin and homobiotin on growth of PRL-W4 on methanol–biotin medium

Compound added	Concentration, μg/liter	Growth after 5 days, Klett–Summerson units*	
		With biotin, 1.0 μg/liter	Without biotin
Norbiotin	0.01	55.9	0
	0.1	64.3	0
	1.0	70.0	0
	10.0	68.0	24.0
Homobiotin	0.01	56.4	0
	0.1	57.3	0
	1.0	70.9	0
	10.0	70.2	0
None	–	49.0	–

NOTE: Medium—Inorganic salts, 0.5% methanol, biotin, and homologues as indicated; 50 ml in 250-ml flask. Culture—Rotary shaker at 30° C.
*See text.

Other Substrates

Since biotin is known to be a cofactor for the aspartase of some organisms (5, 6), the effect of biotin on the cell yield when L-aspartate, fumarate, and succinate were used as substitutes for methanol in the medium was tested. If fumarate were the precursor for aspartate with PRL-W4 and biotin were active as a cofactor for aspartase, it would be expected that the biotin requirement would be less with aspartate in the medium than with fumarate. The results shown in Fig. 5 do not support this postulate since the biotin requirement, while much lower than with methanol as sole carbon source, is about

the same for aspartate, fumarate, and succinate. Aspartate was used as the carbon source in many of the other experiments since growth is faster than with methanol and the medium remains neutral rather than becoming more basic as it does with fumarate and succinate.

Other suitable carbon sources for the growth of PRL-W4 were glutamate, lactate, L-alanine, and glucose. Carbon sources utilized to a lesser extent were fructose, glutarate, and tartrate. Formate, formaldehyde, ethanol, methane, acetate, propionate, oxalate, malate, and glycine were not attacked.

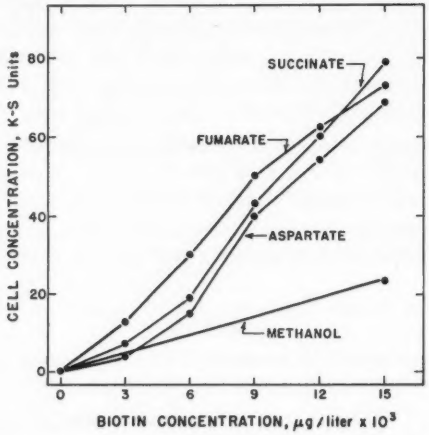

FIG. 5. Effect of biotin concentration of cell yield of PRL-W4 grown on various substrates.
Medium: Inorganic salts, biotin as indicated and one of methanol, 0.5%; L-aspartate, 0.3%; succinate, 0.3%; or fumarate, 0.3%; 50 ml in 250-ml flask.
Culture: Rotary shaker at 30° C.
Maxium cell growth in terms of Klett–Summerson colorimeter units is reported for each culture.

Substrate Concentration

Initial concentrations of methanol in the medium greater than about 1% inhibit the growth of PRL-W4 (Fig. 6). Below this concentration the mean generation time is nearly constant at about 72 hours. Aspartate, fumarate, and succinate inhibited growth at much lower concentrations and were therefore not normally used in media at levels higher than 0.3%.

Respiration

A comparison was made of the effects of formate and methanol on the respiration rates of cells harvested from cultures in which the carbon source was methanol, aspartate, fumarate, or succinate (Table IV). While formate inhibited cultures of growing cells of PRL-W4 at very low concentrations and could not therefore be used as a substrate carbon source, the organism was able to oxidize it in some of these resting cell experiments.

The results obtained (Table IV) indicate that the formate-oxidizing system is present in cells grown on methanol, aspartate, and succinate but not present

in cells grown on fumarate. In contrast, the methanol-oxidizing enzyme system is present only in those cells grown on methanol, since in the other three instances the respiratory rates with methanol in the Warburg flask were lower than the endogenous rates measured with the same preparation of cells.

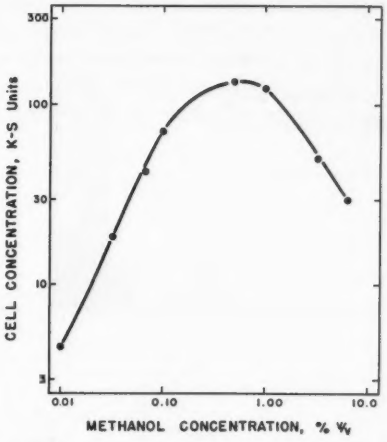

FIG. 6. The effect of methanol concentration on cell yield from cultures of PRL-W4.
Medium: Inorganic salts, 1.0 μg per liter biotin, methanol as indicated; 50 ml in 250-ml flask.
Culture: Rotary shaker at 30° C for 60 hours.

TABLE IV

Effect of substrate carbon source on the ability of PRL-W4 to utilize methanol and formate in resting cell experiments

Carbon source in growth medium		Relative respiration rate*	
		On *M*/100 formate, % of endogenous rate	On *M*/100 methanol, % of endogenous rate
Methanol	0.5%†	135	175
Aspartate	0.3%‡	159	81
Succinate	0.3%‡	138	89
Fumarate	0.3%‡	100	67

*Respiration rates are reported as percentage of the endogenous rate measured for the same cell preparation.
†With 1 μg per liter biotin.
‡With 0.1 μg per liter biotin.

Recovery of Biotin from Cells

Active growth factors can be recovered in good yield from the cells of PRL-W4 grown on media containing biotin, but acid hydrolysis is required (Fig. 7). Mild acid extraction did not recover any detectable amount of growth factor from cells grown on methanol, but some activity (about 10%) appeared in this fraction from cells grown on aspartate. The clarified medium contained no residual growth factor in either case.

The recovered growth factor is primarily biotin as indicated by the position of the activity on the chromatograms (Fig. 7). The lesser amount of activity at an R_f value of 0.45 to 0.50 extracted from the cells grown on the aspartate medium is probably biotin sulphoxide (12). This compound has been reported to occur as an impurity in biotin (13) but the material used in these experiments was chromatographically pure.

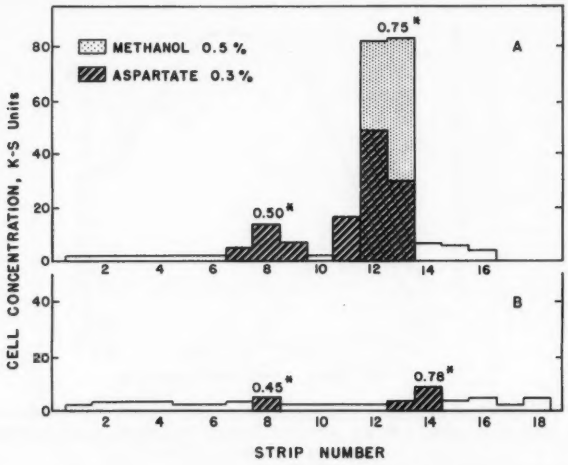

FIG. 7. Growth-promoting activity of eluates obtained from sections of paper chromatograms of acid-soluble and acid-hydrolyzed fractions of PRL-W4 cells.
A. Clarified acid hydrolyzate of cells.
B. Acid extracts of cells.
Medium: Inorganic salts, 1.0 μg per liter biotin, and either 0.5% methanol or 0.3% aspartate as indicated.
Culture: Rotary shaker at 30° C.
*R_f value.

Discussion

There are some similarities between this *Pseudomonas* sp., PRL-W4, and *Pseudomonas methanica*, isolated and described by Dworkin and Foster (3), but important differences exist as well, particularly in the nutritional requirements. Both organisms have a single polar flagellum, produce a water-insoluble red pigment, and have systems for the oxidation of methanol, formaldehyde, and formate. On the other hand, *P. methanica* grew only on methane and methanol among many carbon sources tested, while PRL-W4 grows actively on several carbon sources, in most cases more vigorously than on methanol. Methane is not attacked by PRL-W4 nor can the biotin requirement be replaced by calcium pantothenate.

Since biotin is an essential for cell growth, this organism could be used effectively in a bio-assay method for biotin and related compounds active as

growth factors. A linear relation between the maximum growth rate of PRL-W4 and the initial concentration of biotin in the medium was observed (Fig. 8).

The pathway by which methanol is assimilated by PRL-W4 does not appear to include formate. The enzyme systems for the oxidation of formate are present but growth is not obtained when it is used as the sole source of carbon, even at low concentrations. This apparently rules out the possibility that the organism incorporates carbon mainly from carbon dioxide, obtaining the necessary energy from the oxidation of methanol. It seems very likely that methanol is incorporated in some more reduced form than carbon dioxide or formate.

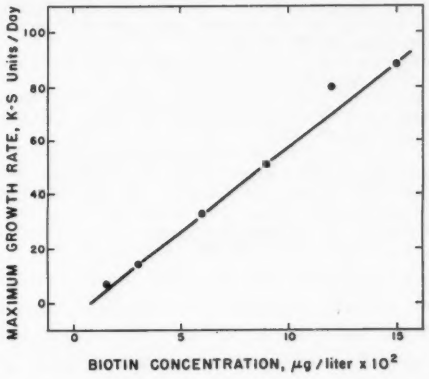

FIG. 8. The effect of initial biotin concentration on the maximum growth rate of PRL-W4.
Medium: Inorganic salts, 0.3% aspartate, biotin as indicated; 50 ml in 250-ml flask.
Culture: Rotary shaker at 30° C.
Maximum rate of growth occurred between 4 and 6 days under these conditions.

The role of biotin in the metabolic pathway of this organism is not established by the results of these experiments. Biotin can be recovered from the cells of PRL-W4 in fair yield, but only by relatively strong acid hydrolysis. More biotin is required for a given cell yield with methanol as the carbon source than with aspartate and some other compounds. Since it has been shown that the ability to use methanol is dependent on adaptive enzyme systems (Table IV) it can be tentatively postulated that these adaptive systems have a requirement for biotin over and above the requirements of the non-adaptive systems active in the utilization of aspartate and other substrates. In some cases biotin plays a part in the bacterial biosynthesis of fatty acids and can be replaced by oleic, pimelic, or azelaic acid. Tween 80 (a non-toxic source of oleic acid, polyoxyethylene sorbitan monooleate), and pimelic acid showed no growth-promoting activity with PRL-W4. The evidence to date indicates a complex requirement for biotin, replaceable only by compounds containing the diaminopelargonic acid skeleton from which the organism can synthesize biotin and possibly biotin sulphoxide.

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MICROBIAL PENTOSANASES

III. SOME FACTORS AFFECTING THE PRODUCTION OF PENTOSANASES BY ASPERGILLUS NIGER AND TRICHODERMA VIRIDE[1]

F. J. SIMPSON

Abstract

Of a number of carbohydrates tested, holocellulose from wheat straw and L-arabinose were the better substrates for production of pentosanase by *Trichoderma viride*. D-Xylose did not induce synthesis of the enzyme by the fungus. Production in a synthetic medium was increased by the addition of malt sprouts, distillers' dried solubles, or glutamic acid.

Under the same conditions the production of pentosanase by *Aspergillus niger* was favored by the water-soluble pentosan of wheat flour, holocellulose, and wheat bran. Both D-xylose and L-arabinose induced synthesis of enzyme. In the synthetic medium the addition of yeast extract, corn steep liquor, malt sprouts, or a number of other nitrogenous adjuncts increased the yield of pentosanase. A medium containing 3% bran ground to pass a 20-mesh sieve, 3% corn steep liquor neutralized with ammonium hydroxide, and 1% calcium carbonate was developed for the production of pentosanase by *A. niger*. Maximum yield was obtained in 60 hours. The pentosanase had an optimum pH of 5.0 and was stable for 30 minutes at 30° C between pH 4.0 and 5.8. The pentosanase could be precipitated from the culture filtrates with 76% ethanol and when stored as a dry powder at 2° C was stable for at least 1 year.

Introduction

In a previous investigation, some factors influencing the production of pentosanases by *Bacillus subtilis* and *Bacillus pumilus* were examined (8). In the present investigation two molds, *Aspergillus niger* and *Trichoderma viride*, were studied. The former produces enzyme when grown on the water-soluble pentosan of wheat flour or D-xylose, while the latter organism, although less active, produces enzyme when grown on the water-soluble pentosan or on L-arabinose, but not on D-xylose (7). For this reason, *T. viride* was included in some of the tests conducted in this investigation.

Materials and Methods

A. niger, PRL 558, and *T. viride* Pers. ex Fries, PRL 198, were stored in sterilized soil and spores were produced on potato dextrose agar. Inoculum was prepared by transferring the spores to 50 ml of medium in a 500-ml Erlenmeyer flask and incubating at 30° C on a Gump rotary shaker (200 r.p.m., $1\frac{1}{4}$ in. eccentricity). The medium consisted of 0.5% carbohydrate, 0.4% Difco nutrient broth, and 5% tomato (homogenized in a Waring blendor) The carbohydrate, which in the preliminary trials was D-xylose for *A. niger* and L-arabinose for *T. viride*, was sterilized separately and aseptically added to the basal medium. In studies concerning the selection and optimum concentration of ingredients for a commercial type medium, the inoculum

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consisted of a culture grown on 1% bran and 1% corn steep liquor adjusted to pH 7.0 with ammonium hydroxide. Growth in 24 hours usually consisted of a mass of mycelia which could be transferred by means of a wide-mouthed pipette. One-half milliliter of this material was used to inoculate 50 ml of test medium in Erlenmeyer flasks of 500-ml capacity.

The basal test medium consisted of 2% carbohydrate, 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.5% $CaCO_3$, and 0.103% of a salt mixture consisting of 1 g $MgSO_4.7H_2O$, 0.01 g $ZnSO_4.7H_2O$, 0.005 g $CuSO_4.5H_2O$, 0.0075 g $MnSO_4.H_2O$, and 0.01 g $FeSO_4.7H_2O$. The usual practice was to sterilize the nitrogenous adjuncts with the basal medium and then aseptically add an aliquot of a sterile solution of the carbohydrate. Carbon sources such as the pectic substances, xylan, holocellulose, straw, bran, water-soluble pentosan of wheat flour, and oat hulls that did not lend themselves to this technique were sterilized with the basal medium. The substrates and nitrogenous adjuncts used in this study were the same as those used previously (8). The flasks containing the test medium were incubated at 30° C on the rotary shaker. Samples of approximately 2 ml were withdrawn at 48 hours and again after 72 hours. The mycelia were removed by centrifugation and the pentosanase activity determined on suitable aliquots of the supernatant liquor. The residual sample was used to determine the pH. Each test was replicated three or four times, the results examined by analysis of variance, and the least significant difference (L.S.D.) calculated from the mean square of the residual error (2, 9). Student's *t* test was also employed (9).

The activity of the pentosanase was determined in the pipette viscometer previously described (7). The reaction mixture consisted of 4.0 ml of a 1.125% pentosan solution, 0.3 ml of a 0.75 *M* acetate buffer (pH 5.0), and 0.2 ml of diluted enzyme. When graded amounts of a culture filtrate of *A. niger* were added to this mixture and held along with suitable controls at

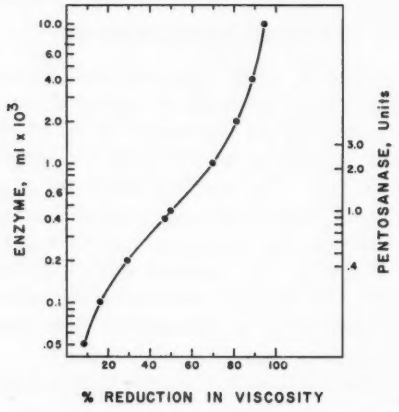

FIG. 1. The relation between the concentration of the pentosanase of *A. niger* and the per cent reduction in viscosity.

30° C for 30 minutes, the percentage decline in viscosity between 30 and 70% was linearly related to the logarithm of enzyme concentration (Fig. 1) (4). A unit of pentosanase was arbitrarily defined as the amount of enzyme that reduced by 50% the viscosity of the standard substrate in 30 minutes at 30° C (7). The samples were assayed for activity on the same day they were taken, but could be stored at 2° C at pH 5 for several days without loss in activity.

The method of Adams and Castagne was used to determine pentosan (1), and the semimicro-Kjeldahl method with a copper–selenium catalyst was used to determine nitrogen.

Experimental

Effect of pH on the Pentosanase of A. niger

The optimum pH was determined in buffers consisting of a mixture of 0.05 *M* glycine, 0.05 *M* succinate, and 0.05 *M* phosphate. The pH of the reaction mixture was determined after the enzymatic activity was measured. The optimum pH is 5.0 (Fig. 2). This is lower than the optimum of 6.0 to 6.5 reported for the xylanase of *Penicillium janthinellum* and *Chaetomium globosum* (10), or of 5.9 reported by Yundt for the xylanase of *A. niger* (11), but higher than that of 4.5 reported for *A. oryzae* (3).

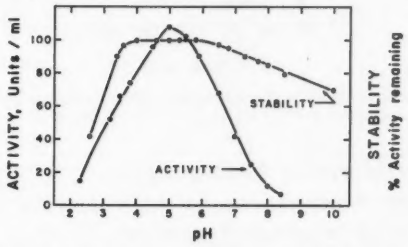

FIG. 2. Activity and stability of the pentosanase of *A. niger* at different pH values.

The stability of the enzyme was determined in 0.5 *M* glycine–succinate–phosphate buffers. Three milliliters of the cultural filtrate was added to 2 ml of 1.25 *M* buffer and held for 30 minutes at 30° C. The pH was determined on an aliquot. After treatment, 1 ml of the mixture was added to 2 ml of 0.5 *M* acetate buffer, pH 5.0. Suitable aliquots were then used to determine the residual enzyme activity. The pentosanase of *A. niger* is stable between pH 4.0 and 5.8 for 30 minutes at 30° C (Fig. 2). Above this range, activity slowly decreases until, at pH 10, 25% of the enzyme was destroyed at 30° C. Below pH 4.0 the enzyme is quickly inactivated. Sixty-eight per cent of the enzyme was destroyed at pH 2.6 in 30 minutes at 30° C.

Effect of Temperature on Production of Pentosanase by A. niger

A New Brunswick shaker (200 r.p.m., 1 in. eccentricity) was used to determine the optimum temperature for production of pentosanase. The shaker, except for the motor, was enclosed in a temperature-controlled box. The

cultures were grown in 25 ml of medium contained in 250-ml Erlenmeyer flasks. At intervals triplicate flasks were removed, water added to compensate for that lost by evaporation, and the pH and pentosanase determined. The maximum amount of enzyme was produced at 35° C (Fig. 3). Either the enzyme is relatively stable at 35° C or stability is not a major factor in determining the optimum temperature for production. Acid production appeared to be greater at the lower temperatures and the pH dropped to 4.0 at 26° C. At 40° C the pH values ranged from 4.5 to 6.0. Although the optimum temperature for production of pentosanase was 35° C, subsequent tests were conducted at 30° C on the Gump shaker that was also being employed for other investigations.

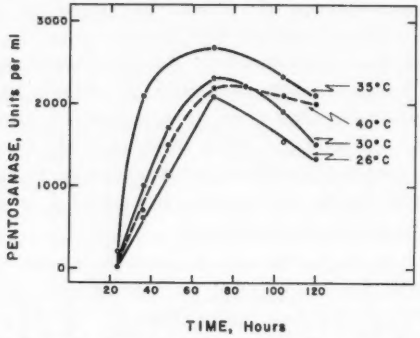

FIG. 3. Effect of temperature on the production of pentosanase by *A. niger*. Medium: 2% xylose, 0.5% yeast extract, 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.5% $CaCO_3$, and 0.103% salt mixture.

Effect of Aeration on the Yield of Pentosanase

For the production of pentosanase, *A. niger* does not appear to have as critical a requirement for oxygen as *B. pumilus*. Whereas the use of volumes larger than 50 ml per 500-ml Erlenmeyer flask resulted in lower yields with *B. pumilus* (8), volumes up to 200 ml were satisfactory for *A. niger* (Table I). Since the pH of the cultures grown in 50 ml of medium dropped to a lower level than those grown in 200 ml, manipulation of the amount of aeration might aid in controlling the pH of the fermentation.

TABLE I

Yields of pentosanase obtained from *A. niger* in Erlenmeyer flasks of 500-ml capacity

Volume	pH		Pentosanase, units/ml	
	48 hours	72 hours	48 hours	72 hours
50	4.5	4.2	2240	1780
75	4.8	4.4	2260	1830
100	6.0	4.9	2400	2290
200	6.2	6.0	2280	2700
300	6.1	6.0	588	1711
L.S.D. ($P = 0.05$)			360	220

NOTE: Medium—3% bran, 2% corn steep liquor, 1% $CaCO_3$ (initial pH 7.0).

Comparison of Carbon Sources

Like that of *B. pumilus*, the pentosanases of *A. niger* and *T. viride* are adaptive enzymes. Whereas *A. niger* produced pentosanase when grown on L-arabinose or D-xylose, *T. viride* responds only to L-arabinose (Table II). This suggests that *T. viride* may produce an L-arabinosidase type of enzyme, and that *A. niger* may produce both an L-arabinosidase and a D-xylosidase. Since the yield of enzyme obtained from *A. niger* with L-arabinose as the inducing agent is considerably less than that produced when D-xylose is the inducing agent, it is likely that two distinctive enzymes are produced rather than a single non-specific type. Subsequent studies on the specificities of the purified enzymes may prove or disprove this hypothesis.

TABLE II

Comparison of carbohydrates for the production of pentosanase

Carbohydrate	Pentosanase, units/ml*	
	A. niger	*T. viride*
None	0	0
Arabinose	227	1000
Xylose	1490	6
Sodium pectate	14	70
Flour pentosan	2860	570
Xylan	839	2
Wheat straw	693	147
Straw holocellulose	2780	2770
Wheat bran	2000	570
Oat hulls	254	69
Beet molasses	68	1
Sulphite waste liquor	620	360

NOTE: Medium—2% carbohydrate, 0.4% $(NH_4)_2HPO_4$, 0.05% ammonium lactate, 0.1% KCl, 0.5% $CaCO_3$, and 0.103% salt mixture.
*Means of three replicates, maximum yields.

The holocellulose of wheat straw, wheat bran, and the water-soluble pentosan were good substrates for the production of pentosanase by *A. niger*. *T. viride* produced pentosanases only on those substrates containing arabinose or polymers of arabinose. Of these, straw holocellulose and L-arabinose were the outstanding substrates. The water-soluble pentosan of wheat flour and wheat bran were less effective. Neither organism produced pentosanase when grown on the following sugars: ribose, fructose, glucose, mannose, galactose, sodium galacturonate, cellobiose, maltose, sucrose, or pectin.

Comparison of Nitrogenous Supplements

The production of amylase by *A. niger* and of pentosanase by *B. pumilus* is favored by complex sources of nitrogen (6, 8). Shu and Blackwood observed that ammonium acetate and protein hydrolyzates were superior to undegraded proteins as sources of nitrogen for *A. niger* (5, 6). The higher yields obtained were attributed to both the nutritive properties and ability to control the pH of the medium. Similarly, although the addition of inorganic salts such as ammonium phosphate, ammonium chloride, or urea to the synthetic medium

increased the amount of pentosanase obtained from *A. niger*, production was greatly favored by the addition of ammonium acetate, casein hydrolyzate, and casitone (Table III). Yeast extract, corn steep liquor, and malt sprouts, however, were even superior nitrogenous adjuncts. Since the pH in all flasks did not drop lower than 4.2, the effect of the adjuncts in this experiment is attributed to their nutritive properties. In addition, in the presence of ammonium phosphate, the protein hydrolyzates, such as casein hydrolyzate and casitone, were not more effective than isoelectric casein.

TABLE III

Effect of nitrogenous adjuncts on the yield of pentosanase after 72 hours of growth

Adjunct	Concentration, %	Pentosanase, units/ml	
		A. niger	*T. viride*
None	–	144	427
$(NH_4)_2HPO_4$	0.24	895	556
NH_4Cl	0.19	402	400
KNO_3	0.36	723	360
Urea	0.11	923	300
(NH_4) acetate	0.28	1430	7
(NH_4) lactate	0.47 (by vol.)	1080	680
(NH_4) citrate	0.40	476	506
(NH_4) succinate	0.27	674	315
Glutamic acid	0.52	700	1500
Casein hydrolyzate	4.76 (by vol.)	1300	2
Casitone	0.46	1270	630
Isoelectric casein	0.35	1010	536
Phytone	0.625	970	700
Soybean meal	0.78	1500	766
Gelysate	0.32	900	747
Gelatin	0.36	1450	421
Peptone	0.33	990	333
Yeast extract	0.53	2860	908
Corn steep liquor	1.23	2780	530
Distillers' dried solubles	1.19	1700	1410
Malt sprouts	1.04	2040	2050
L.S.D. ($P = 0.05$)		265	201

NOTE: Medium—2% carbohydrate, 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.5% $CaCO_3$, 0.103% salt mixture, and nitrogenous adjunct equivalent to 0.5 g N per liter. (Carbohydrate for *A. niger* was D-xylose and for *T. viride*, L-arabinose.)

The addition of nitrogenous materials to the basal medium affected the production of pentosanase by *T. viride* less than that observed for *A. niger*. *T. viride* is sensitive to acetate, and, as a result, growth and the production of pentosanase was poor in the presence of ammonium acetate and casein hydrolyzate. Again malt sprouts were an excellent supplement, but corn steep liquor was decidedly inferior in contrast to the results obtained with *A. niger*. Glutamic acid appears to be an important requirement for the production of pentosanase by *T. viride*.

Effect of Bran Treatments

Wheat bran was selected as the most suitable substrate for the production of pentosanases by *A. niger*. Since most of the hemicellulose of wheat bran is in a water-insoluble form, the bran was ground in a Wiley mill in an effort

to make the substrate more available to the organism (8). Significant increases in yield of pentosanase were obtained when the bran was ground to pass a 20-mesh screen, but retained on a 40-mesh screen as previously observed with *B. pumilus* (8).

Extraction of the bran with alkali or hydrolysis with acid did not result in higher yields of enzyme. On the other hand, these treatments were not detrimental as was observed with *B. pumilus* (8). The treatment with alkali and acids may have destroyed a number of growth factors present in the bran and required by *B. pumilus*, whereas these factors apparently are not required by *A. niger*.

A Medium for the Production of Pentosanase by A. niger

Selection of Ingredients

A medium suitable for the production of pentosanase on a large scale was desired. Wheat bran ground in a Wiley mill to pass a 20-mesh sieve was selected as the carbohydrate and corn steep liquor as the most suitable nitrogenous adjunct. The initial medium contained 2% wheat bran, 2% corn steep liquor, 0.4% dibasic ammonium phosphate, 0.1% potassium chloride, 0.5% calcium carbonate, and 0.103% of the salt mixture. When bran was omitted from the medium, only 50 units of pentosanase were obtained. Bran was therefore an essential ingredient and was present in all media in subsequent tests.

When ammonium phosphate, potassium chloride, and the salt mixture were omitted singly or together, the yield of pentosanase did not differ significantly from that of the complete medium (Table IV). Withdrawal of calcium carbonate alone resulted in an increased yield indicating that calcium carbonate was toxic, but, on the other hand, in the absence of the other inorganic salts, calcium carbonate was required to maintain the pH above 4.0. The use of ammonia as a neutralizing agent in place of calcium carbonate would likely result in higher yields particularly since neutralization of the corn steep liquor with ammonium hydroxide resulted in significantly higher ($P < 0.01$) yields than with sodium hydroxide.

The ingredients of the medium for large-scale production of pentosanase thus contained wheat bran ground to pass a 20-mesh sieve, corn steep liquor neutralized with ammonium hydroxide, and calcium carbonate.

TABLE IV

Effect of omitting ingredients from the tentative medium on yield of pentosanase at 60 hours

Ingredient omitted	Mean yield, $\bar{x}$	$\bar{x}$-Complete medium
None (complete)	2670	–
$CaCO_3$	3000	+330
$(NH_4)_2HPO_4$	2910	+240
KCl	2690	+20
Trace salts	2690	+20
Corn steep liquor	1720	−950
$(NH_4)_2HPO_4$, KCl, salts	2580	−90
$CaCO_3$, $(NH_4)_2HPO_4$, KCl, salts	2220	−450
L.S.D. ($P = .05$)		300

Optimum Concentration of Ingredients

In the presence of 3% bran and 3% corn steep liquor, the optimum concentration of calcium carbonate for the production of pentosanase by *A. niger* was found to be 1% (Fig. 4). In the presence of 3% bran and 1% calcium carbonate, the optimum concentration of corn steep liquor was 1%. When using 3% corn steep liquor and 1% calcium carbonate the optimum concentration of bran for the production of pentosanase was 3% as compared with an optimum of 6% for *B. pumilus* (8). The final medium thus contained 3% bran, 3% corn steep liquor, and 1% calcium carbonate.

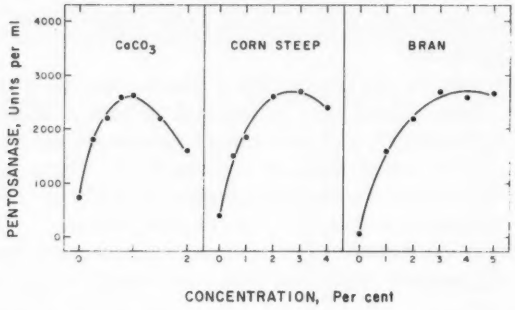

FIG. 4. Relation between concentration of ingredient and production of pentosanase by *A. niger*.

Production Curve

The production of pentosanase and the pH of the fermentation were followed over a period of 120 hours at a temperature of 30° C. Four flasks were removed from the Gump rotary shaker at intervals and the water lost by evaporation was restored. The pentosanase activity and the pH were determined and the means are recorded in Fig. 5. Only trace amounts of enzyme were found in the medium between 0 and 18 hours. The amount of pentosanase produced then rose rapidly to a maximum at 50 to 60 hours. Thereafter the amount of pentosanase in the medium declined. As observed in a

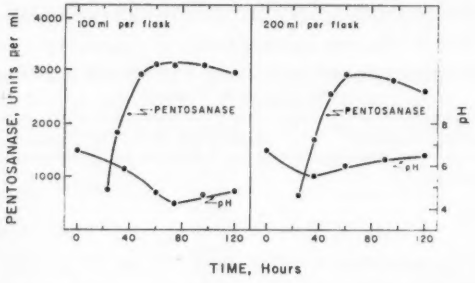

FIG. 5. Production of pentosanase by *A. niger* at 30° C in Erlenmeyer flasks of 500-ml capacity. Medium: 3% wheat bran, 3% corn steep liquor, and 1% calcium carbonate.

previous experiment, the pH of the cultures in those 500-ml Erlenmeyer flasks containing 100 ml of medium dropped to lower values and remained at a lower level than the pH in those flasks containing 200 ml of medium.

The medium has been used to produce pentosanase by *A. niger* in 30-liter stainless steel fermentors with slightly higher yields than those obtained in shake flasks. The enzyme was recovered by removing the insoluble matter in a Sharples supercentrifuge, precipitating the enzyme at 10° C with 4 volumes of ethanol, and then drying the precipitate under high vacuum. The pentosanase in the resulting dry powder is very stable. One sample has been stored for 1 year at 2° C without loss in activity.

Acknowledgment

Grateful acknowledgment is made to Mr. A. S. Sieben for his technical assistance.

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SALICYLATE AS INTERMEDIATE IN THE BREAKDOWN OF AROMATIC RING BY PSEUDOMONAS CONVEXA VAR. HIPPURICUM[1]

MAYA G. BHAT, T. RAMAKRISHNAN, AND J. V. BHAT

Abstract

Pseudomonas convexa var. *hippuricum* cells harvested from hippurate medium were shown to possess the ability to oxidize hippurate, benzoate, salicylate, and catechol without lag phase. Acetone-dried preparations of these cells were found to possess enzymes for oxidizing these substrates. Several *Pseudomonas* strains were demonstrated to have the capacity to use salicylate as a sole source of carbon and energy. In the light of the results obtained on the utilization of benzoate, salicylate, and catechol by *P. convexa* var. *hippuricum* strain grown on those substrates, it is suggested that salicylate is an intermediate in the breakdown of benzoate.

Introduction

Stanier (10), using the technique of simultaneous adaptation, formulated a theory for the microbial oxidation of aromatic compounds, e.g. benzoic acid, by way of catechol and β-ketoadipic acid. He ruled out one of the most obvious possibilities, viz. attack by the successive introduction of phenolic groups on the ring of benzoic acid, as he found that the monohydroxy derivatives of benzoic acid are either not utilized by the bacterial strains he had employed or showed an adaptive lag phase when used with benzoate-grown cells. He therefore concluded that monohydroxybenzoic acids are not in the pathway of benzoic acid breakdown and that, for the first intermediate, two hydroxy groups are simultaneously introduced in the aromatic ring resulting in the formation of catechol.

In our studies on the growth requirements of *Pseudomonas convexa* var. *hippuricum*, strain HPP-3, we found that it could use hippurate, benzoate, as well as salicylate (*o*-hydroxybenzoic acid) as substrates. Catechol was detected after 48 hours in the medium when hippurate was the initial substrate, but it disappeared after 96 hours showing that catechol was being simultaneously formed and utilized and hence was an intermediate in the breakdown of hippurate. In view of the fact that, unlike *P. fluorescens* A.3.12, our pseudomonad strain could utilize salicylate (*o*-hydroxybenzoic acid), it was considered worth while to investigate the oxidation of benzoate by the latter organism and see whether salicylate was in fact in its pathway. Our results using the technique of simultaneous adaptation (9) and acetone-dried cells show that it is so.

Materials and Methods

Materials

The following substances, secured through commercial sources and manufactured by firms mentioned against each, were used in this investigation:

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Contribution from the Fermentation and Pharmacology Laboratories, Indian Institute of Science, Bangalore, India.

hippuric acid (E. Merck, Darmstadt); benzoic acid (British Drug House); glycine (General Biochemicals, Inc.); catechol, recrystallized from toluene (Riedelde Haen A-G); salicylic acid (Bayer's); glucose (Baker's and Adamson's). β-Ketoadipic acid was obtained through the courtesy of Dr. D. K. Banerjee, Professor of Organic Chemistry at this Institute, in the form of diethyl-β-ketoadipate and was used after hydrolysis and recrystallization by the method of Bardhan (1).

Pseudomonas Species

Several *Pseudomonas* strains were isolated in this laboratory from soil enrichments made with hippurate as the sole source of carbon and nitrogen for aerobic growth at 24 to 26° C. The cultures have been tentatively labelled as *Pseudomonas convexa* var. *hippuricum* inasmuch as although, by and large, they resemble *P. convexa* described in the *Bergey's Manual* (2), they differ from it in several minor respects and what is more important in being able to show good growth on hippurate, benzoate, salicylate, and catechol. Glucose, however, serves only as a poor source of carbon for growth and energy. The strain used throughout this investigation was *P. convexa* var. *hippuricum* HPP-3 and this was, like the rest, maintained all along on a synthetic medium containing hippurate and of the following composition in grams per liter of distilled water: neutral phosphate mixture ($Na_2HPO_4 + KH_2PO_4$), 1; NaCl, 0.05; $MgSO_4.7H_2O$, 0.50; $FeSO_4.7H_2O$, 0.01; $CaSO_4.2H_2O$ (saturated solution), 5 ml; micronutrient solution,* 1 ml; hippuric acid, 5; the pH of the medium was adjusted between 7 and 7.2 with NaOH after incorporation of bromocresol purple, which also helped in detecting changes in pH after autoclaving.

Resting Cells

Resting cell preparations were made by centrifuging off the cells from the synthetic medium incubated aerobically at 24 to 26° C and containing 0.5% hippurate, 0.1% benzoate, 0.1% salicylate, or 0.3% glucose as the carbon sources. Catechol-adapted cells were obtained by conducting the adaptation in Warburg vessels as described by Stanier and Tsuchida (11). When hippurate was contained in the medium, no other nitrogen source was incorporated; in other cases 0.05% ammonium sulphate was used. The cells were harvested after 36 hours of growth when they were still in their logarithmic growth phase. The centrifuged cells were washed twice in *M*/30 phosphate buffer and resuspended in phosphate buffer before use or storage in the refrigerator. The cells were at all times used within 4 hours of their preparation especially in view of the observation that the cells kept for a longer period showed a tendency to lose their power to utilize the intermediates.

Methods

Various tests were performed to discover the intermediate products formed when the culture medium was inoculated with the pure culture. The culture medium showed, after 24 hours of growth, clear evidence of hydrolysis of

* Grams per 100 ml distilled water: $ZnSO_4.7H_2O$, 1.1; $MnSO_4.H_2O$, 0.5; $CoSO_4$, 0.005; H_3BO_3, 0.005; Na_2MoO_4, 0.2; $CuSO_4.5H_2O$, 0.0007.

hippuric acid into benzoic acid and glycine. A 10% solution of ferric chloride in water gave buff-colored precipitate indicating the presence of benzoic acid whereas the control hippurate medium (uninoculated) gave no such precipitate. The presence of glycine was detected by chromatographic technique (5). After 48 hours the culture medium was proved to contain catechol by the 'sensitive color test' recommended by Evans (4).

Measurement of Oxidation

Aerobic oxidation experiments were carried out in the conventional Warburg vessels (13) using KOH papers, air as the gas phase and at a temperature of 30° C, in a total volume of 3 ml. Substrate was always tipped in from the side arm in concentration of 5 micromoles (μM). Evolution of CO_2 was measured by the two-vessel method, with and without KOH. Blanks have been subtracted from all figures shown in this paper.

Acetone-Dried Cells

These were prepared by the method recommended by Youmans *et al.* (16) by adding thick homogeneous suspensions of the cells in 0.01 *M* phosphate buffer into 10 volumes of ice-cold dry acetone and grinding the washed material with phosphate buffer to make a fine suspension.

Results

Utilization of Hippuric Acid and its Derivatives

As may be seen from Table I, *P. convexa* var. *hippuricum* could utilize readily hippurate, glycine, benzoate, and salicylate as substrates for growth, while it could not utilize catechol. The oxidizability of catechol was tested manometrically and it was observed that the organism showed oxygen uptake in its presence. Other isolates from the hippurate enrichments, viz. strains HPP-1 through HPP-16, a strain of *P. fermentans*, as also a strain of *P. fluorescens* 8729 obtained from the National Collection, Poona, were also tested

TABLE I

Growth of *Pseudomonas* strains in compounds that lie in the pathway of hippurate metabolism

Organisms	Hippurate	Glycine	Benzoate	Salicylate	Catechol
**P. convexa* var. *hippuricum*					
Strains HPP-1 to HPP-16	+	+	+	+	−
P. aeruginosa					
Strain HPP-17	+	+	+	+	−
Strain PD	+	+	+	+	−
Strain S_2	+	+	+	+	−
Strain PB_5	+	+	+	+	−
P. fluorescens					
Strain 8729	+	+	+	+	−
P. fermentans					
Strain h_1	+	+	+	+	−
Strain h_2	+	+	+	+	−
P. desmolyticum					
Strain HPP-18	+	+	+	−	−

* These strains have been found to differ from each other by virtue of their ability to ferment glycerol and/or reduce nitrate, but essentially belong to the same species. The 16 strains were derived from 16 different soils set up in enrichment cultures on 16 different occasions.

Legend: +, growth; −, no growth.

for their ability to grow on hippurate and its breakdown products in order to see whether salicylate-oxidizing capacity of *P. convexa* strain HPP-3 was peculiar to that strain. It was found that all strains with the exception of *P. desmolyticum* could utilize salicylate for multiplication (Table I). This latter strain, however, when tested in the Warburg apparatus with salicylate as the substrate, it was interesting to observe, could utilize salicylate without any lag period.

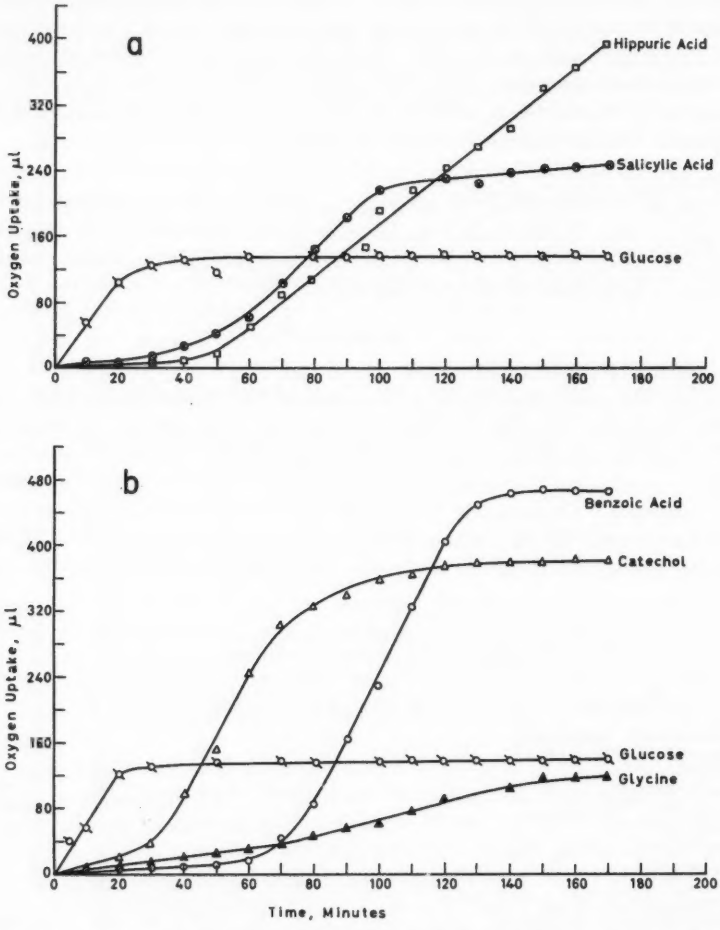
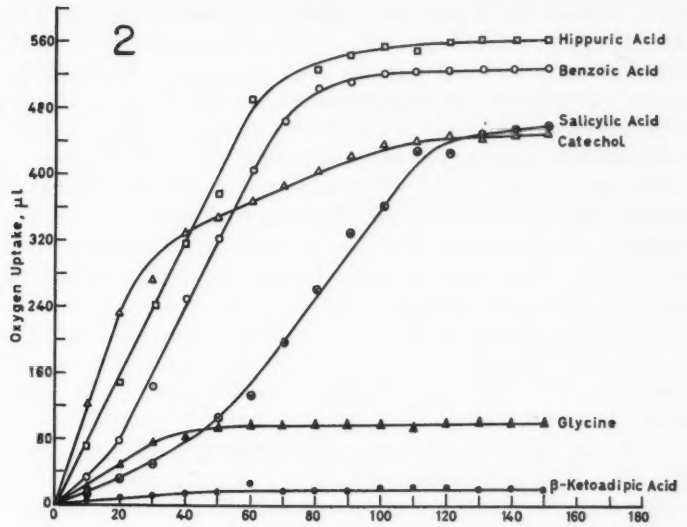


FIG. 1*a*. Oxygen uptake from the oxidation of glucose, hippuric acid, and salicylic acid by washed intact cells grown on glucose. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 μM of substrates.

FIG. 1*b*. Oxygen uptake from the oxidation of glucose, benzoic acid, catechol, and glycine by washed intact cells grown on glucose. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 μM of substrates.



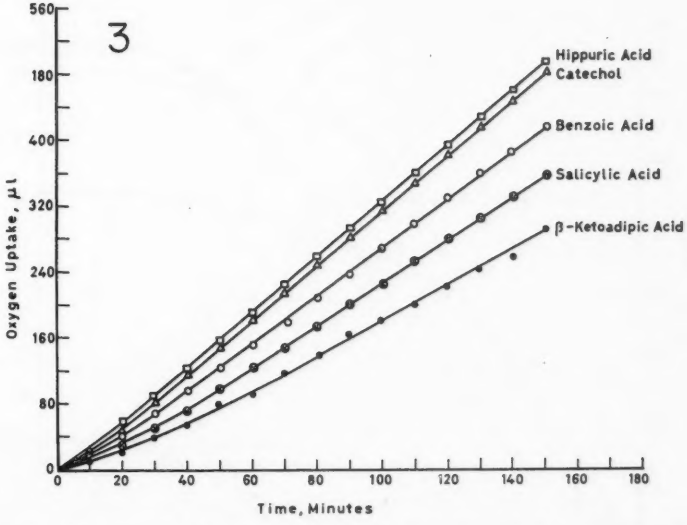


FIG. 2. Oxygen uptake from the oxidation of hippuric acid, benzoic acid, salicylic acid, catechol, glycine, and β-ketoadipic acid by washed intact cells grown on hippuric acid. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 µM of substrates.

FIG. 3. Oxygen uptake from the oxidation of hippuric acid, benzoic acid, salicylic acid, catechol, and β-ketoadipic acid by acetone-dried cells grown on hippuric acid. Components: *M*/15 phosphate buffer, pH 7.0; acetone-dried cells, 0.5 ml; 5 µM of substrates. The value 180 on the ordinate is an error and should read 480.

When *P. convexa* var. *hippuricum* strain was grown on glucose, hippurate, benzoate, salicylate, and catechol were adaptively attacked by it as evidenced by the marked lag in the oxygen uptake (Figs. 1*a* and 1*b*). On the other hand, when grown on hippurate, the organism displayed the ability to attack immediately benzoate, salicylate, glycine, and catechol with oxygen uptake (Fig. 2). β-Ketoadipate was, however, not oxidized possibly due to the non-permeability of the substrate into the cell (7). That non-permeability was indeed the cause was subsequently confirmed by the observation that the acetone-dried cell preparations of the organism could take up oxygen in the presence of β-ketoadipate (Fig. 3). These acetone-dried cells were also able to oxidize hippurate, benzoate, salicylate, and catechol in the same experiment. From the simultaneous adaptation theory of Stanier it can be presumed that the three compounds benzoate, salicylate, and catechol lie in the pathway of hippurate oxidation since all of them were attacked only adaptively by the cells grown on glucose but were immediately oxidized by the hippurate-grown cells. What is more, cells grown on benzoic acid attacked immediately salicylate and catechol, showing that the substrates are in fact in the pathway of benzoate breakdown (Fig. 4). Cells grown on salicylate, on the other hand, though they could utilize catechol without any lag phase, could oxidize benzoate only

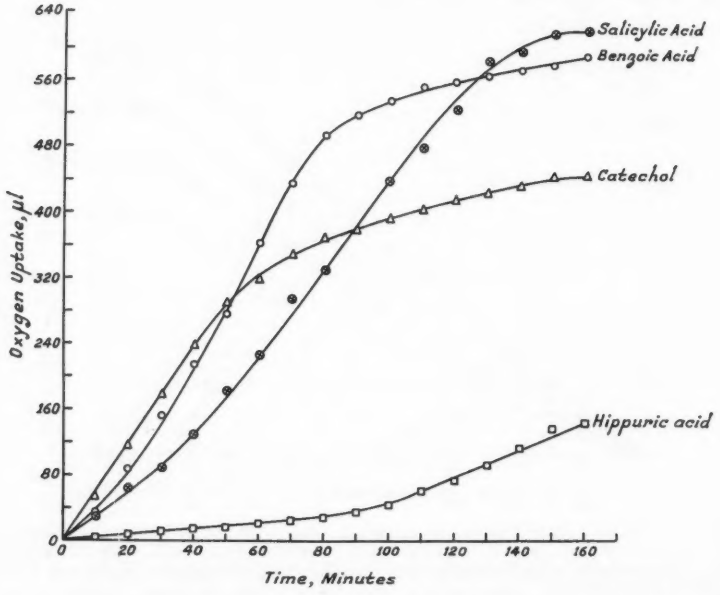


FIG. 4. Oxygen uptake from the oxidation of hippuric acid, benzoic acid, salicylic acid, and catechol by washed intact cells grown on benzoic acid. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 µM of substrates.

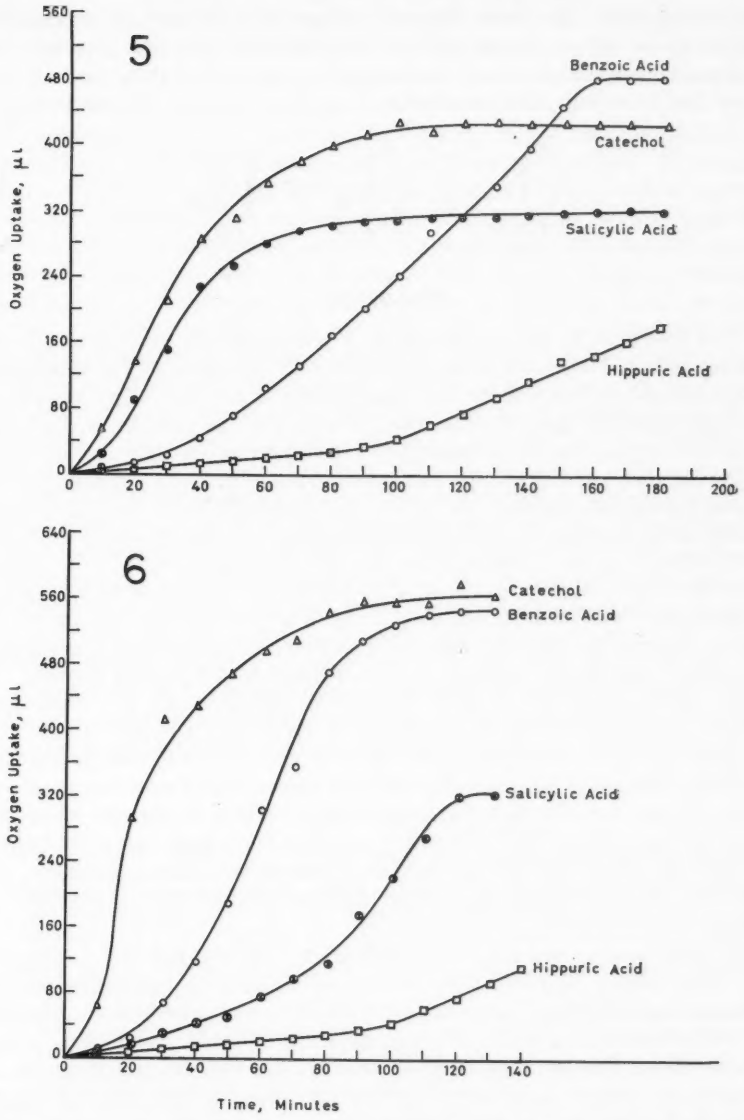
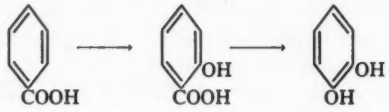


FIG. 5. Oxygen uptake from the oxidation of hippuric acid, benzoic acid, salicylic acid, and catechol by washed intact cells grown on salicylic acid. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 μM of substrates.

FIG. 6. Oxygen uptake from the oxidation of hippuric acid, benzoic acid, salicylic acid, and catechol by washed intact cells adapted to catechol in the Warburg itself. Components: *M*/15 phosphate buffer, pH 7.0; cell suspension, 100 mg wet weight; 5 μM of substrates.

after an adaptive lag phase (Fig. 5). Likewise, cells adapted to catechol failed to utilize both benzoate and salicylate before a lag phase (Fig. 6). All these experimental results lead one to the conclusion that there exists a hippurate–benzoate–salicylate–catechol pathway in *P. convexa* var. *hippuricum*.



Discussion

The fact that 23 out of 24 strains of pseudomonads investigated could grow on salicylate seems to indicate that the salicylate pathway is widespread in this species. What is more, even the solitary strain that failed to grow on salicylate possessed the ability to take up oxygen in the presence of that substrate without any lag phase whatsoever, showing thereby that while it could not use the compound as a carbon source for cell multiplication, it nevertheless possessed enzymes necessary for salicylate utilization under certain circumstances, as for example, when grown on hippurate. Instances where an organism can oxidize certain substances but are unable to use them for growth have been reported in literature. Stanier and Tsuchida (11) showed that some *Pseudomonas* strains oxidize catechol in the Warburg flask but are unable to grow on catechol as the only carbon source owing to the fact the degradation products of catechol in solution act as inhibitors of bacterial growth. A similar reaction may take place in the case of salicylate also and inhibit the growth of an organism like *P. desmolyticum*. Further, it is well known that salicylate uncouples oxidative phosphorylation and though certain strains may get adapted for using salicylate as an energy source, others may not be able to do so. In any case the fact that acetone-dried cells of *P. convexa*, which are grown in hippurate and have lost their viability, have been shown to possess enzymes capable of oxidizing salicylate confirms the finding based on the technique of simultaneous adaptation that salicylate is in the pathway of hippurate oxidation.

The problem as to how benzoic acid is initially attacked has been an obscure one. The finding that salicylate is an intermediate in the breakdown of benzoate clears this up inasmuch as the formation of salicylate entails only the introduction of one hydroxyl group adjacent to the carboxyl group of the benzene ring. Stanier suggested (8) that during the direct conversion of benzoate to catechol two hydroxyl groups are simultaneously introduced into the benzene molecule. In such an event, he postulated, the most likely intermediates could either be 2,3- or 3,4-hydroxybenzoic acid, but both of these have been ruled out by him from the possible intermediates in the oxidation of benzoate by *P. fluorescens*. Evidence in favor of salicylic acid as an intermediate is also available from similar studies carried out on the decomposition of naphthalene and phenanthrene by Gram-negative bacteria (including

pseudomonads) isolated from soil enrichments made with these cyclic compounds (6, 12, 14, 15). If salicylate is an intermediate in the decomposition of napthalene and phenanthrene it is logical that a simpler aromatic compound like benzoate is also degraded via the salicylate pathway.

The successive introduction of hydroxyl groups on the benzene molecule to yield salicylic acid first and then catechol appears more likely. It remains, however, to be seen whether the removal of the carboxyl group of salicylic acid is accomplished in one or two steps. With this end in view, work has been started on the isolation of enzyme (enzymes) responsible for this conversion in *P. convexa* var. *hippuricum* strain. It is difficult to say at this stage why our results are different from those of Stanier (10) since we do not have with us *P. fluorescens* A.3.12 or A.3.8 for comparative studies. We may, however, point out that the lines of evidence adduced by Stanier (10) to show that salicylic acid is not in the pathway of benzoate breakdown by the pseudomonads investigated by him are dependent on the metabolic behavior of intact cells in the presence of externally supplied salicylate. Campbell and Stokes (3) have shown since then that although the resting cells of *P. aeruginosa* 9027 could not oxidize many members of the citric acid cycle, on drying, the very same cells could do so, the limiting factor in the immediate utilization of these intermediary products being the permeability of the cell membrane. It is conceivable, therefore, that the cell walls of certain pseudomonads are impermeable to salicylate and that is the reason why the technique of simultaneous adaptation fails to show salicylate, as an intermediate, in the breakdown of benzoate in every instance studied. The fact that acetone-dried cell preparations of *P. convexa* var. *hippuricum* were able to oxidize β-ketoadipic acid shows that this organism follows a general pattern of decomposition of aromatic compounds as formulated by Stanier.

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NOTES

A NEW METHOD FOR GELATIN LIQUEFACTION*

DONALD M. MYERS†

The value of the gelatin liquefaction test in the bacteriology laboratory has been underestimated primarily because of the time involved in performing the test. Certainly the gelatinase activity as applied to the differentiation of *Aerobacter cloacae* and *Klebsiella pneumoniae* is important. Also, the test is invaluable in the differentiation of *Pseudomonas*, *Alcaligenes*, *Proteus*, *Serratia*, the Arizona group, and their variants.

Present methods using gelatin to demonstrate organisms that elaborate proteolytic enzymes have certain drawbacks. The gelatin "stab" method requires long incubation periods and evidence of liquefaction is demonstrable only after refrigeration of the culture following incubation. The method proposed by Frasier (2) using a plate technique incorporated a low-peptone agar medium to which gelatin was added. Evidence of liquefaction was dependent upon treating the inoculated plates with acid mercuric chloride or tannic acid. The method of Frasier (2) was modified by Clark (3) to increase the sensitivity of the method using $HgCl_2$ as the indicator of liquefaction. These methods require, in most instances, the use of more than one inoculated plate in order to test different time intervals.

The procedure described by Kohn (3) is rapid and may be checked daily, if necessary, for evidence of gelatinase activity. The method depended upon denaturing gelatin in the presence of charcoal with formalin. Evidence of liquefaction would be shown by the release from the gelatin of charcoal particles which would appear as sediment in the tube. An obstacle with this method in our laboratory was the difficulty in obtaining complete removal of the formalin from the denatured gelatin. Trace amounts interfered with the growth of the organism tested. Satisfactory test results were obtained by modifying the technique to include the treatment of the denatured gelatin strips with ammonia.

This paper reports a simplified, practical method for determining gelatin liquefaction, which eliminates certain obstacles in the above methods and maintains the rapidity of the most recent methods.

The method depends upon the use of thinly pressed gelatin capsules filled with bone charcoal, the latter merely acting as an indicator of liquefaction. The capsules used were size No. 2 or No. 3 pharmaceutical gelatin capsules (Parke Davis & Company).

*The opinions expressed are those of the author and do not constitute an endorsement by the Medical Department of the U.S. Navy.

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The filled capsules were placed in a tray on layers of cotton or gauze so the capsule did not come in contact with each other. The tray was then filled with a 10% solution of formaldehyde and allowed to stand at room temperature for approximately 3 hours. During this period of denaturing the gelatin, it is important that the capsules do not come into contact with each other as they will adhere to one another or to the walls of the container.

Following the 3-hour denaturing period the capsules were removed from the tray while being washed with running water and then placed in a large beaker. The beaker containing the gelatin capsules was then filled with a 1.0% solution of ammonium hydroxide and allowed to stand for 1 hour. During this interval all residual formaldehyde is converted by the ammonia water to inactive hexamine (Urotropin).

The capsules were again washed in running tap water to remove any remaining ammonia. This may be accomplished by incubating the capsule at 56° C for 4 hours, changing the water at intervals.

The capsules were then dispensed in lots of 25 into screw-capped bottles, keyed down with a piece of cotton, and the bottle filled with distilled water.

FIG. 1. A positive reaction is shown by the rupture and sedimentation of the charcoal-filled capsule, as compared with a negative reaction with the gelatin capsule remaining suspended and intact.

The capsules were sterilized by intermittent heating at 56° C on 3 consecutive days. The sterilized capsules may be stored at room temperature and, as needed, a capsule is dispensed into a test tube containing approximately 5.0 ml of nutrient broth (Difco) using sterile forceps. If desired, all the capsules may be dispensed into the broth tubes and stored under refrigeration until used. There was no evidence of deterioration of the denatured gelatin. Denatured gelatin does not melt and may be incubated at the usual temperatures.

The test was performed by inoculating the medium in which the gelatin capsule is suspended and by incubating the culture at 37° C or at the optimal temperature for the organism being tested.

Liquefaction was indicated by the rupture of the thin capsule releasing the charcoal, which settled to the bottom of the tube. In negative reactions the charcoal-filled capsule remained suspended on the surface of the medium (see Fig. 1).

The result of the comparison between the capsule method, Kohn's method, and the "stab" method for gelatin liquefaction are given in Table I. There was agreement in the final results with each of the three methods, but longer incubation periods were required by the "stab" method. However, the capsule method produced the most pronounced effect and eliminated questionable reactions.

TABLE I

Analysis of results comparing the capsule method with Kohn's strip method and the stab method

	Number tested	Capsule method		Kohn's method		Stab method	
		Positive days* 1 2 3	Negative days, 14	Positive days 1 2 3	Negative days, 14	Positive days 1 2 3	Negative days, 14
Aerobacter cloacae	6	1 1 1†	2	– 2 1	2	– 1 1†	2
Alcaligenes species	1	– 1 –	–	– 1 –	–	– – 1	–
Alcalescens-Dispar group	2	– – –	2	– – –	2	– – –	2
Escherichia coli	3	– – –	3	– – –	3	– – –	3
Escherichia freundii	3	– – –	3	– – –	3	– – –	3
Hafnia group	1	– – –	1	– – –	1	– – –	1
Klebsiella pneumoniae	1	– – –	1	– – –	1	– – –	1
Proteus mirabilis	4	– 2 –	2	– 2 –	2	– 1 1	2
Proteus morgani	1	– – –	1	– – –	1	– – –	1
Proteus rettgeri	2	– – –	2	– – –	2	– – –	2
Providence group	2	– – –	2	– – –	2	– – –	2
Pseudomonas aeruginosa	8	8 – –	–	8 – –	–	8 – –	–
Salmonella paratyphi A	1	– – –	1	– – –	1	– – –	1
Salmonella typhosa	1	– – –	1	– – –	1	– – –	1
Salmonella typhimurium	1	– – –	1	– – –	1	– – –	1
Serratia marcescens	4	1 3 –	–	1 2 1	–	– 2 2	–
Shigella boydii	1	– – –	1	– – –	1	– – –	1
Shigella dysenteriae	1	– – –	1	– – –	1	– – –	1
Shigella flexneri	1	– – –	1	– – –	1	– – –	1
Shigella sonnei	1	– – –	1	– – –	1	– – –	1
Staphylococcus aureus	5	– 1 –†	3	– – 1	3	– – 1	4

*The figures under the day columns show the number of cultures which became positive on that day.
†Cultures not shown in this column became positive between the 3rd and 14th day.

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THE ANTIBIOTIC SENSITIVITY IN VITRO OF PASTEURELLA HEMOLYTICA AND FOUR SEROLOGICAL TYPES OF PASTEURELLA MULTOGIDA

J. R. MUYSSON AND G. R. CARTER

Coles (1) reported that the sensitivity to streptomycin of his 3 strains of *Pasteurella hemolytica* fell in the same range as 78 strains of *Pasteurella multocida*. The sensitivity of *P. multocida* to various other antibiotics was reported by a number of investigators (2, 3, 4, 5, 6). To our knowledge the action of antibiotics other than streptomycin on *P. hemolytica* in vitro has not been reported. *P. hemolytica* plays a prominent role in the cause of an important disease, shipping fever or stockyards pneumonia of cattle (7, 8, 9). Its sensitivity to antibiotics is, therefore, of particular interest.

In this study the sensitivity to 10 antibiotics of three serologically homogeneous strains of *P. hemolytica* (10) was compared with that of three strains of each serological type (11) of *P. multocida*.

Antibiotics in their pure form were kindly supplied by commercial laboratories. They were dissolved in tryptose phosphate broth (Difco) and sterilized by filtration through 02 Selas candles. Suitable, twofold dilutions were prepared and were stored in 1.0-ml amounts at −25° C overnight. After thawing, the tubes were shaken and inoculated with one loopful (approximately 10^6 organisms) of a 24-hour broth culture. The tubes were incubated at 37° C for 24 hours. The end points were recorded as the minimal inhibitory concentration (12). The resulst are shown in Table I.

The four serological types of *P. multocida* did not differ significantly in their antibiotic sensitivity. *P. hemolytica* demonstrated essentially the same range of sensitivity as *P. multocida*, except, possibly, in the case of bacitracin where there was a suggestion of a greater sensitivity. Except for bacitracin and viomycin, the antibiotics tested were effective against both species of *Pasteurella*.

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TABLE I

Minimal inhibitory concentrations of 10 antibiotics for 15 strains of *Pasteurella*

	P. multocida												*P. hemolytica*		
	A			B			C			D					
Antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Aureomycin HCl, μg/ml	.4	.8	.8	.8	.8	.4	.8	.4	.8	.4	.2	.8	.8	.8	.4
Bacitracin, μ/ml	50	200	200	50	100	200	100	100	100	50	100	100	25	12.5	12.5
Chloromycetin, μg/ml	.8	.8	.8	.8	.8	.8	.4	.4	.8	.4	.8	.8	.8	.8	.8
Erythromycin, μg/ml	1.6	.8	1.6	.8	1.6	.8	1.6	.8	1.6	1.6	1.6	3.1	.4	.4	.8
Neomycin sulphate, μg/ml	6.2	12.5	12.5	12.5	12.5	6.2	12.5	12.5	25	12.5	12.5	25	12.5	12.5	12.5
Penicillin G, potassium, μ/ml	.2	.4	.4	.1	.1	.2	.4	.2	.4	.2	.2	.2	.2	.2	.2
Polymyxin B, μ/ml	25	6.2	6.2	50	25	12.5	3.1	12.5	12.5	25	12.5	12.5	12.5	1.6	12.5
Streptomycin sulphate, μg/ml	12.5	25	12.5	6.2	6.2	6.2	25	25	25	25	50	25	12.5	12.5	12.5
Terramycin HCl, μ/ml	.8	.8	.8	.4	.8	.8	.4	.4	.4	.4	.8	.8	.4	.8	.4
Viomycin, μ/ml	200	100	200	400	400	400	400	200	400	800	200	400	400	400	400

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